THE EFFECT OF THE QUALITY OF PROTEIN ON THE ESTROUS CYCLE [1]

P. B. PEARSON, E. B. HART AND G. BOHSTEDT

Departments of Agricultural Chemistry and Animal Husbandry, University of Wisconsin, Madison

SIX FIGURES

(Received for publication April 21, 1937)

Aberrations of the estrous cycle due to nutritional deficiencies have been reported by various workers. Fraenkel ('17) reported that an insufficient food supply may lead to amenorrhea and sterility in humans. Evans and Bishop ('22), Marrian and Parkes ('30), and Asdell and Crowell ('35) showed that partial inanition interfered with the normal estrous phenomena in the rat. Working with guinea pigs Loeb ('17) found that underfeeding caused a disturbance of the estrous cycle and a hypotypical condition of the ovaries. This hypotypical condition was characterized by a failure of the follicles to mature and by atresia occurring before the follicles reached medium size.

Only a limited amount of information on the effect of the quality of proteins on the sexual behavior of the animal is available. Courrier and Raynaud ('32) found that when rats were fed a diet containing gliadin as the main source of protein the animals failed to ovulate but remained in anestrus. Estrus was induced by implantation of an hypophysis of a normal rat into an animal that was in anestrus. On the basis of this evidence these workers concluded that lysine was necessary for ovulation. Osborne and Mendel ('12) reported

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

that feeding a diet deficient in lysine does not impair the capacity of the animal for reproduction. Their evidence was, however, based on the performance of a single animal. Recently Pearson ('37) has further investigated the influence of a lysine-deficient diet containing 18% of gliadin, which was the sole source of protein except for the small amount that may have been present in the vitamin addenda. Except for the protein the diet contained all essential ingredients necessary for growth. Rats that were fed this diet remained in anestrus; when a preparation of hexone bases or *d*-lysine dihydrochloride was added to the diet normal estrous cycles were resumed. There was no evidence from this work that lysine possessed a specific stimulatory function in maintaining the normal state of the estrous cycle.

In order to throw further light on the effect of the quality of proteins on the estrous cycle and to deduce information as to whether or not a deficiency of other amino acids may not cause an aberration similar to a lysine deficiency, further investigations along this line were undertaken. The estrous behavior has also been correlated with the histologic picture of the ovary.

EXPERIMENTAL

The diets employed are presented in table 1. Gliadin was prepared by extracting wheat gluten with 70% ethyl alcohol. Reprecipitated casein was used. In order to furnish ample amounts of the B factors a 25% acidulated alcoholic extract of brewers' yeast was evaporated on the dextrin at a level equivalent to 2% of yeast. One-half milliliter of liver extract was fed individually each day. The liver extract was prepared from an anhydrous liver concentrate manufactured by Wilson and Company. The powdered liver was dissolved in 2 volumes of water. Five volumes of ethyl ether and 6 volumes of ethyl alcohol were then added and allowed to stand for several hours with frequent shaking. The solvent was then decanted and the precipitate dissolved in water and made up to a volume so that 4 ml. were equivalent to 1 gm. of the liver powder. This material was allowed to stand for

several hours and then centrifuged. The solution was again made up to volume so that 4 ml. were equivalent to 1 gm. of the original liver powder.

The animals used were fed a stock diet until they reached sexual maturity and presented normal estrous cycles. The stage of the estrous cycle was followed by means of daily microscopic examination of the vaginal smear (Long and Evans, '22). The animals in two of the groups were not changed to the restricted diet until they were 100 days or more of age, while a third group was changed at the second period of estrus, which occurred at approximately 50 days

TABLE 1

Percentage composition of diets

	DIET NO.			
	101-P	102-P	104-P	105-P
Dextrin	89.0	84.0	79.0	84.0
Casein	5.0	5.0	5.0	5.0
Gliadin	..	..	..	5.0
Gelatin	..	5.0	10.0	..
Salts[1]	4.0	4.0	4.0	4.0
Cod liver oil	1.0	1.0	1.0	1.0
Wheat germ oil	1.0	1.0	1.0	1.0
Per cent protein	4.82	9.72	14.62	9.70

[1] Phillips and Hart ('35).

of age. This afforded an opportunity to observe the effect of restricted protein intake on the estrous behavior of animals that were practically mature as compared with animals that were immature in respect to growth. Groups of animals were fed ad libitum on a diet containing 5% of casein until the effect on the estrous cycle had been definitely established. Gelatin or gliadin was then added to the diet and the response of the animals observed.

Supplementary effect of gelatin. A group of six rats 105 days of age was placed on diet 101-P containing 5% of casein. The estrous behavior and the average weight of the animals are presented graphically in figure 1. The weight decrement

starting about the fourth week is believed to be due to the use of a liver extract that was impotent. As the animals regained their weight, when a new preparation of liver extract was fed, they were continued in the experiment. There is no reason to believe that this divergence in weight affected the subsequent behavior of the animals in respct to the estrous cycle when gelatin was added to the diet.

It is recognized that a deficiency of the vitamin B factors may have accentuated the aberrations observed during this

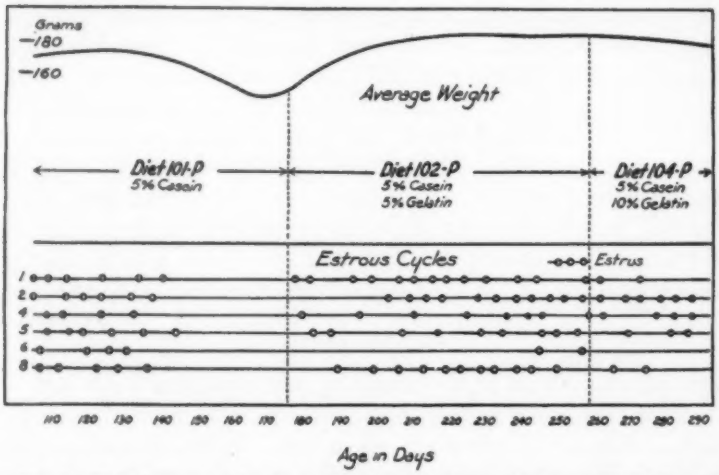

Fig. 1 Weight curve and estrous behavior of rats on diets quantitatively and qualitatively inadequate in protein. The period of estrus is indicated by a circle.

period. In order to clarify this point another group of rats was fed for a period of 10 weeks on diet 101-P. These animals were started on the experimental diet at 110 days of age. Their average weight at the beginning was 190 gm. and after 10 weeks on the low protein diet they weighed an average of 188 gm. All of the animals showed marked aberrations of the estrous cycle after 30 days. In some cases there was complete cessation of estrous cycles, while in others estrus occurred at prolonged and irregular intervals.

Since casein is low in the sulfur bearing amino acids, it might be expected that an addition of cystine to the diet would restore the estrous cycle. However, it has been shown (Pearson, '36) that adding cystine to such a diet was without effect with respect to either the estrous behavior or weight. As it has been demonstrated that lysine is essential for maintaining the normal sexual rhythm we decided to add 5% of gelatin, which is an incomplete protein but relatively high in lysine. According to the analyses of gelatin (Foster and Schmidt, '23; Knoop and Oesterlin, '25) this amount should supply adequate lysine for normal functioning of the animals. After 5% of gelatin was added to the diet none of the animals except no. 2 presented normal estrous cycles. Increasing the gelatin to 10% was without further beneficial effect as there was no significant change in the sexual behavior of the animals after they were transferred to 104-P. At best, the addition of gelatin brings about only a partial response in the estrous cycle. Assuming that the gelatin furnishes ample lysine, there is no evidence that lysine plays a stimulatory role in the sexual phenomena. It does appear, however, that one or more other essential amino acids inhibit normal sexual function. Further evidence to support this postulation is deduced by supplementing a low casein diet with gliadin.

Supplementary effect of gliadin. The animals used in this trial were fed a standard stock diet until the occurrence of the second estrus, at which time they were changed to diet 101-P containing 5% casein. These animals weighed on the average approximately 155 gm. and were considerably younger when placed on the experimental diet than were the animals in the previous group. The performance of these animals is summarized in figure 2 by means of a composite growth curve and a graphical presentation of the estrous cycles. The animals presented one or two normal estrous cycles after being placed on the experimental diet. Subsequently, the periods of anestrus were much longer than normal and in some cases there was complete cessation of the estrous cycle. The animals were continued on diet 101-P for

approximately 70 days, at which time they were changed to diet 105-P which differed from the former diet in that 5% of gliadin was added in place of an equivalent amount of dextrin. It was early demonstrated by Osborne and Mendel ('12, '16) that gliadin is deficient in lysine. On the basis of our knowledge of the amino acid content of gliadin it may be concluded that diet 105-P was not materially higher in lysine than was diet 101-P, but that the content of other essential amino acids was materially increased.

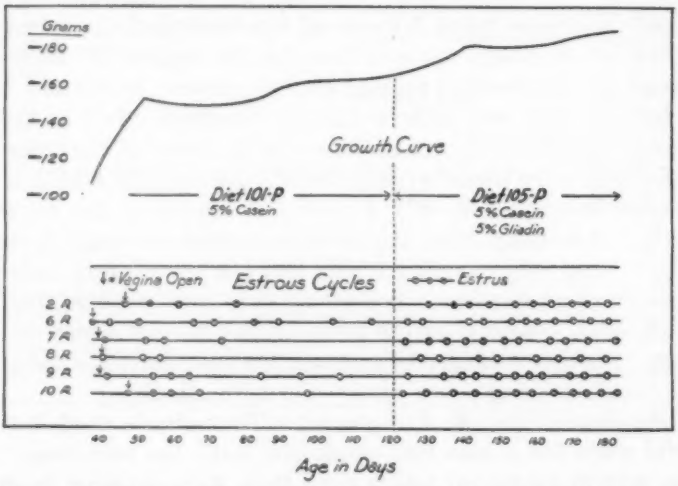

Fig. 2 The effect of adding 5% of gliadin to the diet of rats that have exhibited aberrations of the estrous cycle after being restricted to a diet containing 5% of casein as the sole source of protein. Estrus in indicated by a circle.

From figure 2 it is seen that with the addition of 5% of gliadin to the diet all of the animals passed from anestrus to estrus and continued, thereafter, to present normal estrous cycles. Concomitantly with the resumption of normal estrous behavior there was an increase in weight. The animals were continued on diet 105-P for approximately 62 days. At this time the experiment was concluded and the ovaries were removed for histologic study.

Since there was only a partial response when gelatin, rich in lysine, was added to the diet but when gliadin, low in lysine, was added normal estrous cycles were immediately resumed, it seems apparent that other amino acids are equally as important as lysine in maintaining the normal sexual rhythm, and that a lack of other essential amino acids has a similar effect in causing an aberration of the estrous cycle. We recognize the fact that further work might be done on the effect of the quality of proteins in relation to the estrous phenomena. However, present knowledge appears to warrant the hypothesis that when the dietary protein is inadequate for growth there occurs a disturbance of the estrous cycle and reproductive functions.

Histologic studies. Ovaries from several animals in anestrus and others that had resumed normal cycles after being transferred to diet 105-P were studied histologically in order to correlate the vaginal smear picture with the activity of the ovary, and to observe the changes caused by diets in which the protein is either quantitatively or qualitatively inadequate. The ovaries were fixed in Bouin's fluid, sectioned serially, and stained in hematoxylin and eosin.

The whole reproductive system of the animals that had failed to come into estrus was found to be in a resting or atrophic condition. There were comparatively few primordial follicles; these, together with many of the medium-sized follicles showed various stages of atresia similar to that described by Marrian and Parkes ('30) in partial inanition and vitamin B deficiency, and by Guilbert and Goss ('32) on low protein diets. The most striking feature of the hypotypical ovaries (figs. 3 and 4) was the absence of new corpora lutea. The small shrunken bodies were obviously in the process of involution. The large follicles frequently showed vacuolar degeneration and sloughing off of pycnotic granulosa cells into the antrum. It was evident that ovulation had not taken place during the period of anestrus as recorded by the vaginal smear. In one case the left ovary was removed from a rat that had been on a diet containing 5% of casein and

had been in anestrus 42 days. This was a hypotypical ovary (fig. 4) of the first order with a large amount of interstitial tissue and no large follicles or corpora lutea. The animal came into estrus, as diagnosed by the vaginal smear, 22 days after the laparotomy and then passed into anestrus.

The ovaries of the animals in figure 2, which had showed marked aberration of the estrous cycle when restricted to the 5% casein diet, but which immediately resumed normal estrous cycles when transferred to diet 105-P, appeared normal and active. Large numbers of new corpora lutea were present (fig. 5) and there were many follicles in various stages of development. There was no doubt that ovulation had occurred in a normal manner from these previously degenerate ovaries. In fact, in one animal, 6R, figure 6, an ovum was found in the fallopian tube. From this remarkable recovery of these once degenerate ovaries it is apparent that permanent sterility is not caused by protein deficient regimens, and the follicles are capable of growth and ovulation when the required nutritive stimulus is provided.

From a study of available literature it is apparent that the hypotypical condition of the ovary is similar in all nutritionally inadequate regimens causing cessation of the estrous cycle.

Fig. 3 Photomicrograph of section of ovary of rat no. 8 which had been in anestrus 18 days. The atretic follicles and the absence of new corpora lutea are characteristic of the failure of ovulation due to inadequate nutrition.

Fig. 4 Photomicrograph of section of ovary of rat restricted to diet containing 5% of casein as the main source of protein. This animal had been in anestrus 42 days; the advanced stage of degeneration is characterized by the absence of developing follicles, corpora lutea and excess interstitial tissue.

Fig. 5 Photomicrograph of section of ovary of 6R which had exhibited marked aberration of the estrous cycle on diet 101-P, and then resumed normal cycles when 5% of gliadin was added. The young corpora lutea and normal developing follicles denote ovarian activity and ovulation.

Fig. 6 Photomicrograph of section of oviduct of 6R showing recently extruded ovum.

3
5
6

SUMMARY

Rats maintained on a diet containing 5% of casein as the chief source of protein soon cease to exhibit the characteristic vaginal changes of estrus. The anestrous period is correlated with hypotypical ovaries and atrophic condition of the whole reproductive system. The failure of ovulation is indicated by atretic follicles and the absence of new corpora lutea.

The addition of gelatin, rich in lysine, brings about only a partial response in the estrous cycle. When 5% of gliadin, poor in lysine, is added to the low casein diet ovulation occurs and the normal sexual rhythm is immediately resumed. It is evident that a deficiency of amino acids other than lysine causes an aberration of the estrous cycle, and it seems that when the dietary protein is inadequate for growth the sexual behavior is likewise adversely influenced. No permanent sterility results from feeding diets qualitatively or quantitatively deficient in protein if the necessary stimulus for ovulation is subsequently provided.

LITERATURE CITED

ASDELL, S. A., AND M. F. CROWELL 1935 The effect of retarded growth upon the sexual development of rats. J. Nutrition, vol. 10, pp. 13–24.

COURRIER, R., AND R. RAYNAUD 1932 Modifications génitales chez les animaux à croissance arrêtée par la déficience en lysine. Comp. rend. Soc. Biol., T. 109, pp. 881–882.

EVANS, H. M., AND K. S. BISHOP 1922 On the relations between fertility and nutrition. II. The ovulation rhythm in the rat on inadequate nutritional regimes. J. Metab. Res., vol. 1, pp. 335–356.

FOSTER, G. L., AND C. L. A. SCHMIDT 1923 The separation of the hexone bases from certain protein hydrolysates by electrolysis. J. Biol. Chem., vol. 56, pp. 545–553.

FRAENKEL, L. 1917 Eierstockstätigkeit und Kriegsamenorrhöe. Zbl. f. Gyn., Bd. 41, S. 1033–1037.

GUILBERT, H. R., AND H. GOSS 1932 Some effects of restricted protein intake on the estrous cycle and gestation in the rat. J. Nutrition, vol. 5, pp. 251–265.

KNOOP, F., AND H. OESTERLIN 1925 Über die natürliche Synthese der Aminosaüren und ihre experimentelle Reproduktion. Z. physiol. Chem., Bd. 148, S. 294–315.

LOEB, L. 1917 The experimental production of hypotypical ovaries through underfeeding. A contribution to the analysis of sterility. Biol. Bull., vol. 32, pp. 91–115.

LONG, J. A., AND H. M. EVANS 1922 The oestrous cycle in the rat and its associated phenomena. Memoirs Univ. of Calif., vol. 6.

MARRIAN, F. G., AND A. S. PARKES 1930 The effect of anterior pituitary preparations administered during dietary anoestrus. Proc. Roy. Soc. B., vol. 105, pp. 248–257.

OSBORNE, T. B., AND L. B. MENDEL 1912 The role of gliadin in nutrition. J. Biol. Chem., vol. 12, pp. 473–510.

———— 1916 The amino-acid minimum for maintenance and growth, as exemplified by further experiments with lysine and tryptophane. J. Biol. Chem., vol. 25, pp. 1–12.

PEARSON, P. B. 1936 Dietary protein in relation to the estrous cycle. Proc. Am. Soc. Anim. Prod. Twenty-ninth Annual Meeting, pp. 282–285.

———— 1937 The effect of a lysine deficient diet on the estrous cycle. Am. J. Physiol., vol. 118, pp. 786–791.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon fluorine toxicosis in the rat. J. Biol. Chem., vol. 109, pp. 657–663.

STUDIES ON THE ENERGY METABOLISM OF THE HEN [1]

H. H. DUKES [2]

Veterinary Research Institute, Iowa State College, Ames, Iowa, and Department of Physiology, New York State Veterinary College, Cornell University, Ithaca, New York

FOUR FIGURES

(Received for publication April 7, 1937)

Work on the gaseous exchange of the chicken was begun several years ago by the writer in response to the need, by a group of workers collaborating on a poultry ventilation project, for data on oxygen consumption and carbon dioxide production. Soon after work on the gaseous exchange was initiated, it seemed wise to broaden the studies to include other phases of the energy metabolism of chickens. This paper is concerned chiefly with: the effect of fasting on the energy metabolism, the basal metabolism, the heat loss due to vaporization of water, the relationship between insensible loss and metabolic rate, and the effect of egg production on the metabolic rate.

Since several recent reports on the energy metabolism of the hen (Mitchell and Haines, '27 a, b; Mitchell, Card and Haines, '27; Benedict, Landauer and Fox, '32) contain numerous references to the literature, it is not considered necessary to treat the literature extensively in the present paper. Only those papers having a direct bearing on the problems at hand will be mentioned.

[1] Presented in preliminary form at the Washington meeting of the American Physiological Society, March, 1936.

[2] Now at Cornell University.

METHOD

The Haldane (1892) gravimetric method of measuring the respiratory exchange was used. It was customary to have the water absorbers in pairs. In this way the second bottle could be used to check the efficiency of absorption by the first. The efficiency of the carbon dioxide absorption was checked either by having the absorber bottles in duplicate or by causing the air from the bottles to pass through a solution of barium hydroxide. Blank experiments (twenty-eight in number) indicated that the average error in the oxygen determination was not greater than 2%. Carpenter ('28) has shown by means of alcohol check tests that the Haldane method will give reliable results with adult poultry if the periods are sufficiently long—at least 2 hours. Most of the periods in the present work were 2 hours long.

The preliminary periods were usually ½ hour in duration. The rate of ventilation was kept constant throughout an experiment at 3 liters per minute. This is sufficient to prevent condensation of moisture in the chamber.

The heat production was calculated in the usual way. Determinations of the nitrogen metabolism were not made. Where the respiratory quotient was greater than 1, the carbon dioxide in excess of the oxygen was given a heat value of 1.09 calories per liter (Lusk, '28). Only five tests showed respiratory quotients above unity. Where the respiratory quotient was less than 0.707 but not less than 0.68, a respiratory quotient of 0.707 was assumed in making the calculations. The number of tests in this group was twenty-eight. Where the respiratory quotient was less than 0.68, the test was discarded. Only three tests were rejected for this reason.

The surface area of the birds was estimated according to the well-known Meeh formula ($S = k \times W^{\frac{2}{3}}$), 0.10 being used for the value of k (Benedict, Landauer and Fox, '32).

Activity records were made in most of the tests. The animal chamber was so suspended that when the bird was active the chamber would move up and down at one end. These movements were picked up by a receiving tambour and trans-

mitted to a recording tambour arranged to write on a kymograph. The activity records were divided into three classes depending upon whether the hens were quiet (I), moderately active (II) or active (III). Upon examination of the data it was evident that when the activity had been estimated to be II the metabolic rate was so little increased over the rate when the activity had been estimated to be I that the results could be placed in the same group from the standpoint of activity. Results obtained during periods in which the activity had been judged to be III were rejected (except in the three instances identifiable in table 1). Twenty-two tests were discarded because of an activity of III.

The environmental temperatures in the different experiments ranged from 21 to 29°C. The lowest of these figures is well above the critical temperature of the hen as determined by Mitchell and Haines ('27 a). Most of the experiments came within the temperature range of 23 to 27°C. During a given experiment the temperature range was usually much narrower than this.

Early in the course of this work the practice was adopted of standardizing the amount of food in the crop at the beginning of a fast. This was accomplished by removing the hens from the hen house overnight and the next morning (at which time the crop was usually found to be empty) feeding them 40 gm. of corn. The time elapsing between the feeding of the corn and the middle of the test was considered to be the duration of the fast.

The weighings were done on a 10 kg. Sauter balance having a sensitivity of 10 to 20 mg. depending on the load.

One hundred and fifty-six trials were run. Fifty-four hens (mostly Plymouth Rocks) were used. Only the results in day experiments are reported.

RESULTS

The effect of fasting on the respiratory quotient and the metabolic rate. This question was studied in fifty tests on eight Plymouth Rock hens 10 to 13 months old. The results

are shown in figure 1. It is evident that the average respiratory quotient has fallen to 0.74 by the end of the first day and to 0.70 by the end of the second day. From that time on, it remains practically constant. The average metabolic rate,

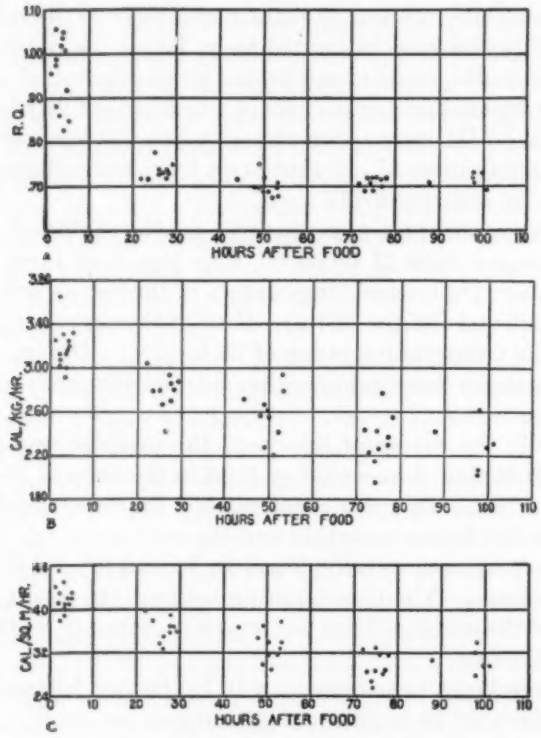

Fig. 1 The effect of fasting on the respiratory quotient and the metabolic rate. Fifty tests on eight Plymouth Rock hens, 10 to 13 months old.

both on a body weight basis and on a surface area basis, shows a progressive fall until about the seventy-fifth hour. From then on, the rate appears to be practically constant.

The basal metabolism. To obtain further data on the respiratory quotient and the metabolic rate after a fast of approximately 24 hours, fifty-four tests were run on thirty-two White

Plymouth Rock hens ranging in age from 18 to 22 months and in weight from 1.95 to 3.28 kg. (average weight, 2.51±0.03 kg.). The fasts were 23 to 32 hours in duration. The results are shown in the accompanying histograms (fig. 2). It is evident that with several distinct exceptions the data are distributed close to the normal.

TABLE 1

The R.Q. and metabolic rate 23 to 29 hours after food
(Plymouth Rock and White Wyandotte hens)

HEN NO.	AGE (MONTHS)	HOURS WITHOUT FOOD	R.Q.	CAL./KG./HR.	CAL./SQ.M./HR. ($s = 0.10 \times w^{\frac{2}{3}}$)	ACTIVITY	HEN NO.	AGE (MONTHS)	HOURS WITHOUT FOOD	R.Q.	CAL./KG./HR.	CAL./SQ.M./HR. ($s = 0.10 \times w^{\frac{2}{3}}$)	ACTIVITY
67	12	25	0.71	2.76	35.51	I	1662	12	23	0.72	2.87	35.88	I
		27	0.72	2.68	34.53	I			25	0.77	2.85	35.62	I
		29	0.70	2.66	34.14	I							
57	12	25	0.75	2.91	34.06	†	44	14	24	0.74	2.27	30.97	I
		27	0.78	2.73	31.92	I			26	0.71	2.22	30.32	I
		29	0.75	2.72	31.70	I			28	0.71	2.23	30.51	I
697	Old	25	0.74	2.18	28.22	II	2430	22	26	0.69	2.36	32.93	II
		27	0.75	2.34	30.12	II			29	0.68	2.15	30.15	I
		29	0.76	2.30	29.62	II							
30	12	24	0.73	2.29	31.15	I	2440	22	24	0.75	2.45	35.94	II
		26	0.71	2.26	30.67	I			26	0.70	2.28	33.25	I
		28	0.73	2.40	32.62	III			28	0.70	2.18	31.93	I
27	12	25	0.73	2.27	29.67	I	1562	22	25	0.79	2.85	38.23	I
		27	0.73	2.33	30.48	I			27	0.77	3.32	44.50	III
		29	0.72	2.33	30.45	I			28	0.73	3.01	40.40	II
23	12	25	0.78	2.59	34.75	I	†	22	24	0.75	2.96	39.85	I
		27	0.80	2.57	34.51	II			26	0.72	3.06	40.10	II
									28	0.72	3.11	41.60	II
†	12	25	0.76	2.38	33.09	I	2479	22	24	0.78	2.21	31.64	I
		27	0.77	2.22	30.80	I			26	0.77	2.14	30.60	I
		29	0.74	2.41	33.44	I			27	0.75	2.18	31.20	I
46	14	24	0.69	3.37	43.41	III	1512	22	25	0.73	2.03	30.59	I
		26	0.73	2.69	34.54	I			28	0.70	2.09	31.03	I
		28	0.72	2.92	37.59	II							

Means: R.Q., 0.74±0.003; cal./kg./hr., 2.53±0.03; cal./sq.m./hr., 33.73±0.39.

To determine whether it is possible to repeat metabolism measurements after a fast of approximately 24 to 30 hours—that is, to determine whether during this interval the metabolic rate remains constant—numerous tests were run with the results shown in table 1. Examination of this table shows that

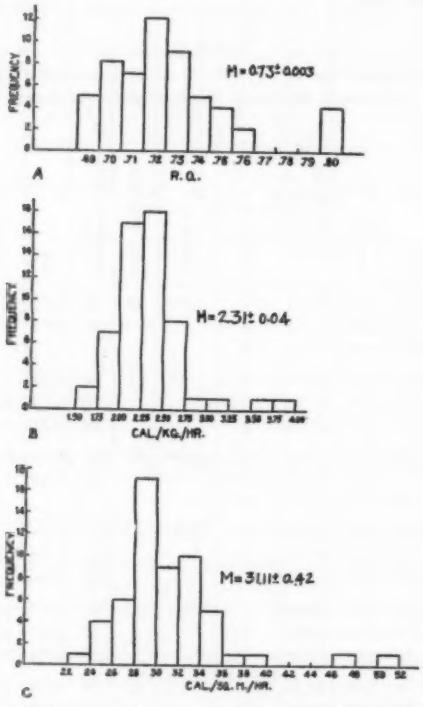

Fig. 2 The respiratory quotient and the metabolic rate of the hen 23 to 32 hours without food. Fifty-four tests on thirty-two White Plymouth Rock hens, 18 to 22 months old.

on the whole, if the activity of the bird is uniform, it is possible during this time to repeat with accuracy metabolic rate determinations on the same hen.

Combining the results on basal metabolism shown in figure 2, B and C, and table 1 (age of hens, 12 months or more), it appears that the average basal metabolism of the mature hen

is not far from 2.4 calories per kilogram per hour and 32.4 calories per square meter per hour, or 57.6 calories per kilogram per day and 778 calories per square meter per day.

The variability of the basal metabolism. The fifty-four basal metabolism tests summarized in figure 2 were made on

TABLE 2

The variability of the basal metabolism (White Plymouth Rock hens, 18 to 22 months old; non-laying)

HEN NO.	DATE OF TEST	WEIGHT	CAL./KG./HR.	CAL./SQ.M./HR.	CHANGE IN METABOLISM (CAL./SQ.M./HR.)	ACTIVITY
		kg.			%	
1594	11/ 2/31	2.26	2.41	31.62		I
	12/30/31	3.04	1.64	23.72	−25	I
1616	11/ 5/31	2.17	2.29	29.73		I
	12/30/31	2.84	1.83	25.95	−13	II
1549	11/ 5/31	2.08	2.57	32.77		I
	1/ 6/32	2.59	2.47	33.95	+ 4	I
1667	11/ 9/31	2.24	2.59	33.84		II
	12/ 1/31	2.40	3.81	51.20	+51	II
	1/ 6/32	2.77	2.36	32.23		I
	1/29/32	3.00	2.05	29.62	− 8	I
2510	11/12/31	2.43	2.38	31.90		I
	12/ 1/31	2.26	2.50	32.80	+ 3	I
1546	11/12/31	2.18	2.71	35.19		I
	1/ 8/32	2.97	2.03	29.18	−17	I
1516	11/17/31	2.65	1.97	27.14		II
	1/20/32	2.72	1.79	24.85	− 8	I
1659	11/20/31	2.56	2.06	28.14		I
	1/22/32	2.73	1.99	27.83	− 1	I
1615	12/ 1/31	2.80	1.74	24.54		II
	1/ 6/32	2.85	1.76	24.75	+ 1	II
2541	12/ 7/31	2.23	2.67	35.00		I
	2/ 3/32	2.58	2.11	29.00	−17	I
2510	1/20/32	2.27	2.18	28.68		?
	2/ 3/32	2.32	2.40	31.57	+10	I

thirty-two hens over a period of several months. Since more than one test was run on a large number of the hens, it is possible to arrange the results derived from these hens in such a way that the question of the variability of the basal metabolism can be studied. This is done in table 2. The data are from

hens that were not laying. It is evident that in several instances the change in the metabolic rate was large.

The heat loss due to vaporization of water. In this method of metabolism measurement, the water elimination of the subject is determined by the gain in weight of the sulphuric acid bottles coming in line immediately after the animal chamber. If during an experiment an egg is laid or feces are eliminated, the vaporization of water would be increased. The water elimination in such an experiment should therefore not be used in a study of the insensible water loss of the birds. Of

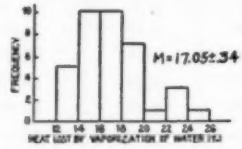

Fig. 3 The percentage of heat lost by vaporization of water. The data are from the basal metabolism studies summarized in figure 2.

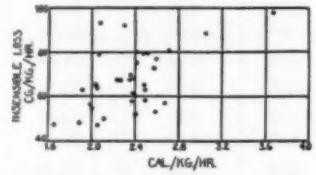

Fig. 4 The correlation between insensible loss and metabolic rate. The data are from the basal metabolism studies summarized in figure 2. The mean insensible loss is 0.675 gm. per kilogram per hour; the mean basal heat production, 2.32 calories per kilogram per hour. The correlation coefficient is 0.411.

the fifty-four tests depicted in figure 2, thirty-seven showed no egg or feces in the chamber. The average insensible water loss in these experiments was 0.68 ± 0.001 gm. per kilogram per hour. The minimum was 0.46, the maximum 0.99 gm. In each of the thirty-seven tests the percentage of heat lost by vaporization of water was calculated, 0.585 calories being assumed to be the amount of heat required to vaporize 1 gm. of water. The results are shown graphically in figure 3. It is evident that the distribution of the data is close to the normal.

Correlation between insensible loss and metabolic rate. It has been shown by Benedict and Root ('26) in man and by Kriss ('30) in cattle that there is a high positive correlation between the total insensible loss of the organism and the

TABLE 3

The effect of egg production on the fasting metabolic rate (White Plymouth Rock hens, 18 to 22 months old)

HEN NO.	DATE OF TEST	WEIGHT	CAL./KG./HR.	CAL./SQ.M./HR.	CHANGE IN METABOLISM (CAL./SQ.M./HR.)	ACTIVITY	EGG PRODUCTION FOR MONTH IN WHICH THE TEST WAS MADE	DAYS BETWEEN WHICH OR ON WHICH EGGS WERE LAID
		kg.			%			
2321	11/ 2/31	2.32	2.41	31.90		II	11	12–31
	1/ 6/32	2.50	2.51	34.15	+ 7	II	13	12–30
2169	11/ 5/31	2.04	2.35	29.83		II	1	25
	12/30/31	2.23	2.50	32.74	+10	I	11	1–31
2197	1/22/32	2.65	2.15	29.45		I	3	8, 24, 27
1699	11/23/31	2.49	2.38	32.43		I	0	
	1/29/32	2.64	2.58	35.60	+10	II	4	6, 24, 25, 30
1453	12/ 1/31	2.28	2.68	35.33		II	0	
	2/ 3/32	2.48	2.78	37.60	+ 6	II	16	1–28
2440	11/ 9/31	3.11	1.91	27.88		I	1	28
	2/10/32	3.17	2.23	32.59	+17	I	13	2–26
1562	11/12/31	2.10	2.24	28.77	+ 3	I	0	
	1/ 8/32	2.46	2.08	27.96		II	0	
	2/11/32	2.44	2.85	38.23	+37	I	11	1–29
2479	12/18/31	2.81	2.02	28.40		I	0	
	2/17/32	2.94	2.18	31.15	+10	I	5	23–29
1512	11/20/31	3.24	2.04	30.17	− 8	I	0	
	1/22/32	3.28	2.02	27.83		I	0	
	2/19/32	3.27	2.06	30.81	+ 4	I	5	20–28
	2/25/32	3.21	2.17	31.97		I		
2504	2/25/32	2.80	2.20	30.95		I	3	24–27

metabolic rate. Many others have worked on the problem (Du Bois, '36, p. 62). A measurement of the total insensible loss being a necessary part of a metabolism test by the Haldane method, data were at hand for a determination of the relationship between the insensible loss and the metabolic rate.

The data are from the experiments incorporated in figure 2, and are therefore basal data. All insensible losses from experiments in which an egg or feces were found in the chamber were discarded for the purposes of this correlation. The correlation is shown by means of a scatter diagram (fig. 4). The correlation coefficient of the two sets of data is 0.411. Fisher's 't' test indicates that the correlation is significant.

The effect of egg production on the fasting metabolic rate. The metabolism tests forming the basis of figure 2 were made over a period of several months. Records of the egg production of the flock were kept during that time. Since several of the hens laid fairly well during the months in which their metabolic rates were determined, it is possible to assemble the data in such a way that the effect of egg production can be studied. This is done in table 3.

DISCUSSION

Mitchell and Haines ('27 b) and Benedict, Landauer and Fox ('32) have pointed out that a fast of at least 48 hours is necessary to bring the hen into the post-absorptive, and therefore the basal, condition. Under the conditions of the present experiments, it is clear that in the interval extending from 24 to 30 hours after food the metabolic rate of the hen is uniform (table 1) and the respiratory quotient is on the average about 0.73 (figs. 1A and 2A; table 1). It is therefore evident that further fasting is not required; and by analogy with man it appears justifiable to speak of the metabolic rate of resting hens without food for 24 to 30 hours as the basal metabolism. The average basal metabolic rate reported here is practically the same as the mean of the averages obtained by Mitchell and Haines ('27 b) and Benedict, Landauer and Fox ('32) in day experiments, and very close to the mean of the averages of the day and night experiments of Benedict, Landauer and Fox.

Figure 2 shows that several of the hens were distinctly aberrant with respect to the fasting respiratory quotient and the metabolic rate. It is worth noting that the hens with the high respiratory quotients were not the ones with the high

metabolic rates. The bird (no. 1667) showing the highest metabolic rate (51.20 calories per square meter per hour) showed a normal rate 3 weeks earlier, and 5 and 8 weeks later (table 2). This brings up the important question of the variability of the basal metabolism. The data in table 2 indicate a fairly constant basal metabolism in most of the hens, but there are several distinct exceptions. Although the majority of the hens gained in weight between metabolism tests, there does not appear to be any correlation between the increase in weight and any change in metabolic rate. The recent work of Benedict and Ritzman ('35) indicates that in dairy cattle individual animals may show very great lability of the basal metabolism. In the hen the lability is not as great as in dairy cattle but it is greater than in man (Du Bois, '36, p. 184).

An age effect on the basal metabolism seems evident from the data here reported. The metabolic rates shown in figure 1 (B and C) after a fast of approximately a day's duration are distinctly higher, on the average, than the rates shown in figure 2 (B and C), where the fast was of about the same duration. The hens in the latter group were 8 to 10 months older than those in the former. It appears therefore that the basal metabolism of the hen decreases with age. Experiments of Mitchell, Card and Haines ('27) and of Mitchell and Haines ('27 b) indicate that the basal metabolism of mature hens is lower than that of pullets, and the work of Brody, Funk and Kempster ('32) shows that the heat production of chickens declines with age.

The percentage of heat lost by vaporization of water in the fasting hen is on the average 17 (fig. 3). This is much lower than the average figure (49%) obtained by Benedict, Landauer and Fox ('32) on a fasting hen and cock. This difference is doubtless explainable by the fact that the average environmental temperature at which the measurements of Benedict and co-workers were made was several degrees higher than the average in the present series. In fact, Doctor Benedict in a private communication to the author states that in experiments on geese, made at a lower environmental temperature

than in their experiments on chickens, the percentage of heat lost by vaporization is much lower. The heat lost by vaporization in man is usually considered to be about 24% of the total heat loss (Du Bois, '36, p. 62), but Burton and Murlin ('35) have reported lower values; 17.8% on the average. The average in the present paper is very close to this.

According to Gerhartz ('14) the basal metabolism of the laying hen is definitely higher than that of the non-laying hen, and Mitchell and Haines ('27 b) report a small amount of evidence to support this view. On the other hand, Brody, Funk and Kempster ('32) did not note, in a limited amount of data, any marked difference between the heat production of good-laying and of poor-laying pullets. Examination of table 3 in the present paper shows in all instances an increase in the basal metabolic rate with the onset of egg production. However, in most hens the increase is small and in several it is of no significance. Nevertheless in at least two hens (2440 and 1562) the increase is large enough to be significant. Of importance also may be the fact that during the same period several of the non-laying birds in the flock showed definite declines in the basal metabolism (table 2). The data as a whole appear to justify the conclusion that egg production is accompanied by some increase in the basal metabolism. Further information is required, however, before a definite statement can be made.

SUMMARY

In a study of the energy metabolism of the hen (156 determinations on fifty-four birds) it was found that:

1. In prolonged fasting the metabolic rate fell progressively until about the seventy-fifth hour. From then on to about the 100th hour the rate appeared to be practically uniform. The respiratory quotient showed no great change after about the twenty-fourth hour (fig. 1).

2. The average basal metabolic rate of mature hens, after a fast of approximately 24 to 30 hours' duration, was not far from 2.4 calories per kilogram per hour and 32.4 calories per

square meter per hour (fig. 2, B and C; table 1), or 57.6 calories per kilogram per day and 778 calories per square meter per day. The average basal respiratory quotient of hens was about 0.73 (figs. 1A and 2A; table 1).

3. During the interval extending from 24 to 30 hours after food the metabolic rate in individual hens was uniform (table 1).

4. Most of the hens showed a fairly constant basal metabolism over a period of 1 to 2 months, but several showed great lability in the basal metabolism (table 2).

5. The basal metabolism was lower in older hens.

6. The heat loss due to vaporization of water (basal) varied from 12 to 25% of the total heat loss and averaged 17% (fig. 3).

7. The basal insensible loss and the basal metabolism showed a positive correlation (fig. 4).

8. Egg production was accompanied by a small increase in the basal metabolism (table 3).

LITERATURE CITED

BENEDICT, F. G., W. LANDAUER AND E. L. FOX 1932 The physiology of the normal and frizzle fowl, with special reference to the basal metabolism. Storrs Agr. Exp. Sta., Bull. no. 177.

BENEDICT, F. G., AND E. G. RITZMAN 1935 Lability of the basal metabolism of the dairy cow. Proc. Nat. Acad. Sci., vol. 21, p. 304.

BENEDICT, F. G., AND H. F. ROOT 1926 Insensible perspiration: its relation to human physiology and pathology. Arch. Int. Med., vol. 38, p. 1.

BRODY, S., E. M. FUNK AND H. L. KEMPSTER 1932 Age changes in the heat production of chickens as measured by a graphic method. A preliminary report. Poultry Sci., vol. 11, p. 133.

BURTON, A. C., AND J. R. MURLIN 1935 Human calorimetry. III. Temperature distribution, blood flow and heat storage in the body in basal condition and after ingestion of food. J. Nutrition, vol. 9, p. 281.

CARPENTER, T. M. 1928 Control tests of a Haldane chamber apparatus in the metabolic study of adult alcoholized poultry. J. Lab. and Clin. Med., vol. 14, p. 73.

DU BOIS, E. F. 1936 Basal Metabolism in Health and Disease. 3rd. ed. Lea & Febiger, Philadelphia.

GERHARTZ, H. 1914 Über die zum Aufbau der Eizelle notvendige Energie (Transformationsenergie). Arch. ges. Physiol., Bd. 156, S. 1.

HALDANE, J. S. 1892 A new form of apparatus for measuring the respiratory exchange of animals. J. Physiol., vol. 13, p. 419.

KRISS, M. 1930 Quantitative relations of the dry matter of the food consumed, the heat production, the gaseous outgo and the insensible loss in body weight of cattle. J. Agr. Res., vol. 40, p. 283.

LUSK, G. 1928 The Elements of the Science of Nutrition. 4th ed., p. 397. W. B. Saunders Co., Philadelphia.

MITCHELL, H. H., AND W. T. HAINES 1927 a The critical temperature of the chicken. J. Agr. Res., vol. 34, p. 549.

———— 1927 b The basal metabolism of mature chickens and the net-energy value of corn. J. Agr. Res., vol. 34, p. 927.

MITCHELL, H. H., L. E. CARD AND W. T. HAINES 1927 The effect of age, sex and castration on the basal heat production of chickens. J. Agr. Res., vol. 34, p. 945.

THE EFFECT OF YEAST ON THE LIVER GLYCOGEN OF WHITE RATS DURING HYPERTHYROIDISM

VICTOR A. DRILL
Department of Biology, Long Island University, Brooklyn

(Received for publication April 8, 1937)

It is generally accepted that a reduction of the liver glycogen in the cat, rat, mouse, guinea pig, or rabbit, can be brought about by feeding desiccated thyroid (Frazier and Friedman, '35; and others). Coggeshall and Green ('33) found that the liver glycogen in rats was reduced most by thyroxin, less by dried thyroid gland, and not at all by inorganic iodides, when these substances were administered in proportion to their iodine content.

In 1931, Himwich, Goldfarb and Cowgill found that dogs fed desiccated thyroid needed more vitamin B complex as judged by the onset of anorexia and loss of weight. This was confirmed in the pigeon by Cowgill and Palmieri ('33).

Sure and Smith ('34) reported that vitamin B (B_1), when fed simultaneously with oral or subcutaneous doses of thyroxin, showed a protective action against the effect of the thyroxin as judged by the loss of weight. Later Sure and Buchanan ('35) reported on the quantitative balance between thyroxin and vitamin B, in which they determined the minimal dose of vitamin B that would still produce a gain in weight while the animals were receiving thyroxin.

The question then arises: If vitamin B will prevent a loss of weight in rats receiving thyroxin, will it also prevent a loss of liver glycogen? Abelin ('30) and Abelin, Knochel and Spichtin ('30) found that large amounts of casein, egg yolk, or yeast in

the diet of hyperthyroid animals would lower the basal metabolic rate and would also prevent liver injury. In their experiment they fed 100, 200 or 300 mg. of thyroid per day for 6 days together with yeast and obtained beneficial results, even though the animals still lost weight. The vitamin content of the yeast is not stated.

It was thought advisable to examine the change in the percentage of liver glycogen, if any, when small amounts of thyroxin were injected daily over a longer period of time, instead of the short 6-day period used by Abelin et al. Yeast, in which the vitamins B and G are known, was to be fed in order to produce a constant weight, or a gain in weight, in rats receiving thyroxin, and the liver of these rats then analyzed for the percentage of glycogen and compared with the control rats.

EXPERIMENTAL WORK

In this experiment dry yeast [1] was used as the source of vitamin B instead of injecting crystalline vitamin B. This was done to eliminate any possible shortage of other vitamins in the vitamin B complex, for if we injected vitamin B and used autoclaved beef as a source of vitamin G we would not be sure that the animals would receive the other essential vitamins in the complex. The yeast used in the experiment contained 18 international units of vitamin B per gram and 20 Sherman units of vitamin G per gram. A daily injection of 0.1 mg. of crystalline thyroxin [2] was given subcutaneously. The following diet (no. 3) was used: Steenbock's no. 40 salt mixture, 4 gm.; cod liver oil, 2 gm.; Crisco, 10 gm.; casein (extracted with dilute acetic acid, followed by 60%, then 90% ethyl alcohol), 20 gm.; starch, 64 gm.

Group 1 (positive controls). These rats received a mixture of 97 gm. of diet no. 3 plus 3 gm. of yeast. This gives 54 international units of vitamin B and 60 Sherman units of vitamin G per 100 gm. of food. No thyroxin injections were given this group. Seven rats (five males, two females) were used. At

[1] Fleischmann's dried yeast, no. 15190.

[2] Squibb's no. 57999.

the beginning of the experiment two males were killed and the liver glycogen determined. The other three males and the two females were continued on the above diet until the end of the experiment. The rats ate 12 to 13 gm. per day. Water was constantly supplied.

Group 2 (test rats). Each rat received 10 gm. of diet no. 3 plus 3 gm. of dry yeast per day, giving each rat 54 international units of vitamin B and 60 Sherman units of vitamin G per day. The 13 gm. of food was consumed nearly every day. Nine rats (five males, four females) were used. Each rat also received 0.1 mg. of thyroxin injected subcutaneously per day. Water was constantly supplied.

Group 3 (negative controls). These rats received the same diet as group 1 plus a daily injection of 0.1 mg. of thyroxin subcutaneously. Eight rats (six males, two females) were used. The rats ate 12 to 13 gm. of food per day. Water was constantly supplied.

EXPERIMENTAL RECORD

9/9/36. The special diets and the daily injections were begun.

9/18/36. The yeast content of the negative and positive controls was reduced to 1.0 gm. of yeast plus 99 gm. of diet no. 3. Note that the reduction was made in both groups 1 and 3. This change was made because the negative controls were still gaining weight at this date. A balance of diet was sought so that group 1 would gain weight on the amount of yeast provided, and so that group 3 (negative controls) would lose weight on the same diet when injected with 0.1 mg. of thyroxin per day. It was then planned, by raising the yeast content, to produce a gain in weight in group 2 which was also receiving thyroxin.

9/21/36. Rat no. 89 in group 2 died. (See table 2.)

9/22/36. Rat no. 88 in group 3 died. (See table 3.)

9/27/36. The weights of the negative controls had dropped varying degrees. The positive controls and the test rats continued to gain in weight. All of the rats were killed and the liver glycogen determined by a modification of the Pfluger

method by Good et al. ('33). The sugar obtained in this analysis was then determined by the method of Shaffer and Somogyi ('33). Forsgren ('29) has shown that there is a functional variation in the liver glycogen content. All of the rats were therefore fed at the same time each day to produce the same cycle of glycogen variation in each rat; and the rats were all killed 24 hours after the last injection and feeding and all of the livers were dissected from the rats within 1 hour, limiting, therefore, any cyclic variation in the glycogen to a minimal change.

TABLE 1

Group 1 (positive controls)

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN
				%
73	M	Both rats killed at beginning		2.17
74	M	of experiment		2.34
		gm.	*gm.*	
76	M	144	177	3.74
83	M	143	162	2.63
84	M	151	185	2.67
80	F	120	139	2.56
81	F	99	138	2.50
			Average	2.65

RESULTS

The rats in group 1 (positive controls) all made a gain in weight. The values for the liver glycogen are normal and check with one another. Table 1 shows the weights of the rats and the results of the analysis.

The test rats in group 2 had not lost weight by the end of the experiment. Three of the rats remained constant in weight (nos. 86, 87, 90) and the other five rats made a considerable gain in weight. All of the liver glycogen percentages are normal in range, the average being slightly below the rats of group 1. The results of the analysis and the weights of the rats are shown in table 2.

The rats in group 3 (negative controls) received the same diet as group 1, which contained enough yeast to produce a

steady gain in weight, as evidenced by the growth of the rats in group 1. The rats in group 3 were then made to lose weight by the injection of 0.1 mg. thyroxin per day. Two rats (nos. 82 and 57) did not have a loss of weight at the end of the experiment, although their weight fluctuated during the experiment. The percentage of these two rats is close to the normal values in table 2. The results obtained on these two rats are therefore discarded and the negative control values are based on the remaining five rats, all of which show the low liver glycogen percentages of hyperthyroidism. The results of this group are

TABLE 2

Group 2 (test rats)

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN	COMMENT
		gm.	*gm.*	%	
86	M	164	163	2.41	Weight remained constant
87	M	154	154	2.29	Weight remained constant
90	M	166	165	1.86	Weight remained constant
89	M	156	106	...	Died 9/21/36
58	F	115	125	1.99	Gain in weight
59	F	123	142	2.29	Gain in weight
78	F	113	137	2.23	Gain in weight
79	F	107	119	2.39	Gain in weight
92	M	140	156	2.02	Gain in weight
			Average	2.18	

tabulated in table 3. In table 3 the column headed 'Highest weight recorded' means the highest weight that these rats showed, before the sudden effects of the injections caused hyperthyroidism and subsequent loss of weight. Group 2 was protected against the hyperthyroidism by the slightly higher vitamin content of the diet, and no high-point in the weight is then obtainable, because the rats made a constant gain in weight.

Table 4 is included to show the effect of severe hyperthyroidism on the liver glycogen of rats, when fed 0.1 gm. of thyroid gland each day for 12 days. Table 5 shows more normal values for the liver glycogen in rats which were in the same series as those in which severe hyperthyroidism was produced (table 4).

The rats in group 1 (positive controls) receiving 2.1 to 2.4 international units of vitamin B and 2.4 to 2.6 Sherman units of vitamin G per day, gained weight. The negative controls in group 3, receiving the same amount of vitamin, lost weight when injected with thyroxin and glycogen per cent in the liver was less than in group 1. In the test rats (group 2), each rat

TABLE 3

Group 3 (negative controls)

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	HIGHEST WEIGHT RECORDED	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN	COMMENT
		gm.	*gm.*	*gm.*	%	
60	M	151	155	136	0.68	
75	M	131	137	113	0.83	
77	M	132	143	127	1.14	
85	M	131	152	123	0.28	
88	M	142	152	117	...	Died 9/22/36
91	M	130	132	123	1.27	
57	F	105	113	111	1.63	Weight remained constant
82	F	111	119	119	1.56	Results not averaged (see text)
				Average	0.84	

TABLE 4

Severe hyperthyroidism

RAT NO.	SEX	WEIGHT AT START OF EXPERIMENT	WEIGHT AT END OF EXPERIMENT	LIVER GLYCOGEN
		gm.	*gm.*	%
47	M	156	113	0.16
46	M	172	131	0.18
48	F	145	115	0.26
55	M	171	154	0.06

TABLE 5

Normal rats (concurrent with table 4)

RAT NO.	SEX	DATE KILLED	LIVER GLYCOGEN
			%
50	F	All rats were killed	2.95
51	F	6/27/36 and had made	2.05
53	M	a steady gain in weight	1.63
56	M	before death.	1.89

received about 54 units of vitamin B and about 60 units of vitamin G per day and did not lose weight or liver glycogen when injected with the same amount of thyroxin as group 3. This demonstrates that when yeast is fed in an amount sufficient to prevent a loss of weight in hyperthyroid rats, a loss of liver glycogen can also be prevented, keeping the percentage of liver glycogen very close to that of normal animals.

DISCUSSION

In the literature a number of conflicting papers have appeared on the relationship between the vitamin B complex and liver glycogen. Ariyama ('33) found that the liver glycogen of pigeons increased during vitamin B deficiency, but under fasting conditions the liver glycogen was less than normal. Abderhalden and Wertheimer ('32) found an accumulation of glycogen in the liver during vitamin B deficiency. Westenbrink ('33) reported that the liver could form glycogen normally during vitamin B deficiency.

On the other hand, Labbé and associates ('33 a) found that vitamin B favors the deposition of glycogen in the liver and reduces glucemia and glycosuria. They also reported ('33 b) that the vitamin B complex favored the formation of glycogen in the liver, and also increased the glutathione content of the liver. Sure and Smith ('32) using rats that received the same number of calories as the vitamin B complex deficient rats, found a lowered liver glycogen in the vitamin deficient rats. Collazo and Bayo ('31) found a lowered glycogen content in B avitaminosis and also that yeast autolyzate would cure these pathological conditions, whereas insulin would produce the same beneficial results as the yeast without, however, curing the vitamin deficiency. He therefore concluded that the nutritional disturbances of vitamin B avitaminosis are not due to a pancreatic dysfunction, but are related to carbohydrate metabolism. Lajos ('36) found that vitamin B aids the storage of sugar as liver glycogen and acts similarly to insulin, but more slowly, in reducing blood sugar. The weight of evidence appears to be in favor of the view that vitamin B aids the storage of glycogen in the liver. This is substantiated by the work

of Abelin and associates and by the experiment performed above.

Westenbrink and Overbeek ('33) report that in the absence of vitamin B the resorption of glucose is lessened, and Osuka ('31) found that in normal animals yeast only promotes glycogen formation if the diet contains 1 to 2 gm. of sugar per day. Frazier and Friedman ('35) found a lower percentage of glycogen in the liver during hyperthyroidism, which could not be raised by the feeding of sugar.

If the lowering of the liver glycogen by vitamin B deficient diets is substantiated in future research, it is suggested that the loss of glycogen in the liver during hyperthyroidism might be due to the destruction (or loss of use) of the vitamin B (or B complex) by thyroxin or desiccated thyroid. The low liver glycogen might therefore be due to vitamin B deficiency; the vitamin B deficiency being brought on by the hyperthyroidism. This would be borne out by the work of Sure and associates in stopping a loss of weight, in rats receiving thyroxin, with vitamin B, and by Abelin et al., and by this experiment on liver glycogen during hyperthyroidism. It would also explain why Frazier and Friedman could not raise the liver glycogen during hyperthyroidism with sugar; because the vitamin B (or B complex) was destroyed or its function inhibited by the thyroid hormone.

CONCLUSIONS

1. A group of rats on a normal diet containing 2.1 to 2.4 international units of vitamin B (B_1) per day and 2.4 to 2.6 Sherman units of vitamin G per day gained in weight and showed normal liver glycogen values.

2. A group of rats on a normal diet containing 2.1 to 2.4 international units of vitamin B and 2.4 to 2.6 Sherman units of vitamin G and receiving 0.1 mg. of thyroxin subcutaneously per day, eventually lost weight and showed low liver glycogen values.

3. A group of rats on a normal diet containing 54 international units of vitamin B and 60 Sherman units of G and

receiving 0.1 mg. of thyroxin subcutaneously per day, still gained or remained constant in weight and showed normal liver glycogen values.

4. The change in the vitamin content of the diet of the test rats, as regulated by the amount of yeast, is responsible for the normal liver glycogen value.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Ralph Holt Cheney of Long Island University for his interest and counsel in this work. The crystalline thyroxin was supplied through the courtesy of Dr. John F. Anderson of E. R. Squibb and Sons, and the yeast through the courtesy of Mr. Robert F. Light of the Fleischmann Laboratories.

LITERATURE CITED

ABELIN, I. 1930 Effect of casein in hyperthyroid metabolism. Biochem. Zeits., Bd. 228, S. 165.

ABELIN, I., M. KNOCHEL AND W. SPICHTIN 1930 The effect of vitamins on the course of experimental hyperthyroidism. Biochem. Zeits., Bd. 228, S. 189.

ABDERHALDEN, E., AND E. WERTHEIMER 1932 Über das Verhalten des Glykogen im Organismus bei Abwesenheit des Vitamin B-Komplexes (insbesondere des vitamins B_1) in der Nährung. Arch. f. d. ges. Physiol., Bd. 230, S. 601.

ARIYAMA, TSUNE 1933 Quantitative change of glycogen in the liver in vitamin B_1 deficiency. J. Agr. Chem. Soc. Japan, vol. 9, p. 1394.

COGGESHALL, H. C., AND J. A. GREENE 1933 Influence of desiccated thyroid gland, thyroxin, and inorganic iodine upon the storage of glycogen in the liver of the albino rat, under controlled conditions. Am. J. Physiol., vol. 105, p. 103.

COLLAZO, J. A., AND C. P. BAYO 1931 Influence of the B vitamins and of insulin on the disturbances of carbohydrate metabolism in vitamin B deficiency. Biochem. Zeits., Bd. 238, S. 335.

COWGILL, G. R., AND M. L. PALMIERI 1933 The effect of experimentally induced hyperthyroidism on the vitamin B requirement of pigeons. Am. J. Physiol., vol. 105, p. 146.

FORSGREN, E. 1929 Anatomical qualities of liver during various stages of its functional activities. J. Morph. and Physiol., vol. 47, p. 519.

FRAZIER, W. D., AND H. FRIEDMAN 1935 Alterations in liver glycogen following thyroid, iodine, and glucose feedings. Surg., Gyn. and Obst., vol. 60, p. 27.

GOOD, C. A., H. KRAMER AND M. SOMOGYI 1933 Determination of glycogen. J. Biol. Chem., vol. 100, p. 485.

HIMWICH, H. E., W. GOLDFARB AND G. R. COWGILL 1931 Vitamin B complex in relation to food intake during hyperthyroidism. Proc. Soc. Exp. Biol. and Med., vol. 28, p. 646.

LABBÉ, M., F. NEPVEUX AND J. D. GRINGOIRE 1933 a Le role des Vitamines B dans le metabolisme hydrocarbone. Leur emploi dans le traitement du diabete. Bull. acad. de med., T. 109, p. 689.

———— 1933 b Influence des vitamines B sur la teneur en glycogene et en glutathion du foie des lapins. J. de med. de Paris, T. 53, p. 501, also Compt. rend. soc. biol., T. 113, p. 152.

LAJOS, S. 1936 Effect of vitamin B on carbohydrate metabolism. Biochem. Zeits., Bd. 284, S. 279.

OSUKA, T. 1931 Die regulatorische Wirkung der Hefe auf die einseitige Ernährung von Ratten. Biochem. Zeits., Bd. 239, S. 163.

SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determinations. J. Biol. Chem., vol. 100, p. 695.

SURE, B., AND K. S. BUCHANAN 1935 Vitamin B_1 and thyroxin. Proc. Soc. Exp. Biol. and Med., vol. 33, p. 77.

SURE, B., AND M. SMITH 1932 The effect of a deficiency of the vitamin B complex on the lipid metabolism and the glycogen contents of the liver of the albino rat. Arch. Int. Med., vol. 49, p. 397.

———— 1934 Hyperthyroidism and nutrition; vitamin B and thyroxin. J. Nutrition, vol. 7, p. 547.

WESTENBRINK, H. G. 1933 Vitamin B und Leberglykogen. Arch. neerl. de physiol., Bd. 18, S. 429.

WESTENBRINK, H. G., AND G. A. OVERBEEK 1933 Vitamin B und Darmresorption. Arch. neerl. de physiol., Bd. 18, S. 416.

EFFECT OF ADDING COPPER TO THE EXCLUSIVE MILK DIET USED IN THE PREPARATION OF ANEMIC RATS UPON THEIR SUBSEQUENT RESPONSE TO IRON

MARGARET CAMMACK SMITH AND LOUISE OTIS
Department of Nutrition, University of Arizona, Tucson

TWO FIGURES

(Received for publication April 12, 1937)

The necessity of copper as a supplement to iron for hemoglobin synthesis was first demonstrated by Hart et al. ('28). Since that time the mode of action of copper has been investigated (Joseph, '32; Elvehjem and Sherman, '32) and it has been shown that copper does not affect the assimilation of iron but functions in the conversion of iron to hemoglobin. Thus stored iron can only be utilized for synthesis of hemoglobin when copper is available.

In the recommended procedure for the experimental production of anemic rats for use in studies on availability of iron, however, this effect of copper has received no consideration. Rats are used which have been placed upon a whole milk diet until the hemoglobin concentration of the blood falls to a certain low level (2 to 5 gm. per cent) at which time exhaustion of iron reserves is taken for granted. The hemoglobin regeneration resulting from the subsequent feeding of a food is then taken as a measure of the amount of available iron in that food.

Elvehjem has stressed the necessity for exhaustion of body iron reserves in preparation of the test animals for studies of availability of iron in foods, for unless this is done, it is obvious that the hemoglobin synthesis which follows the

feeding of a food will be a measure of the copper content of that food as well as its available iron. Schultze and Elvehjem ('33) have reported that a "slight initial rise of hemoglobin and erythrocytes" results from the feeding of copper alone to anemic rats.

It seems logical to believe that a fall in hemoglobin to a low level in rats on a whole milk diet may occur even in the presence of tissue iron reserves, because of lack of adequate copper to catalyze its conversion into hemoglobin, and therefore that low hemoglobin values in animals on a milk diet regime can only be used as criteria of exhaustion of this store of iron if ample copper has been provided to insure its utilization. Accordingly, the effect of adding copper to the milk diet used for production of experimental nutritional anemia has been further investigated and the response to iron supplementation of rats made anemic in the presence of copper has been measured.

EXPERIMENTAL PROCEDURE AND RESULTS

Following the essentials of the Elvehjem-Kemmerer technic ('31) for the production of anemia, rats were weaned at 3 weeks of age and continued upon whole milk as their sole food. It has been previously found that the hemoglobin of rats handled in this fashion in this laboratory reached a concentration of 4.5 gm. per 100 cc. or below in 4 to 5 weeks. In these experiments, therefore, at the end of the fourth week each rat was given 0.05 mg. copper as copper sulfate daily for 1 to 2 weeks in addition to the whole milk diet in order to insure the presence of ample copper for the conversion of all residual iron into hemoglobin, and graded portions of iron as ferric chloride supplemented with copper and manganese were then given. The gains in hemoglobin of these rats in the following 6-week test period were measured and compared with the hemoglobin regeneration of rats which received the same amounts of iron but which were not given supplemental copper in the anemia preparation period. Details of the housing and care of these rats, method of giving

the supplements and hemoglobin measurement have been describd in a previous paper (Smith and Otis, '37 a).

Figure 1 shows graphically the effect of adding copper alone to the milk diet of anemic rats. Whereas, the hemoglobin concentration in the blood of the milk fed rats which were not given copper supplements fell steadily, the mortality being high beyond the eighth week, it may be seen that the addition of copper resulted in an increase in hemoglobin. The initial rise in hemoglobin concentration was, however, usually followed by a fall to approximately the original level and the decline in hemoglobin thereafter was slight, if any. This

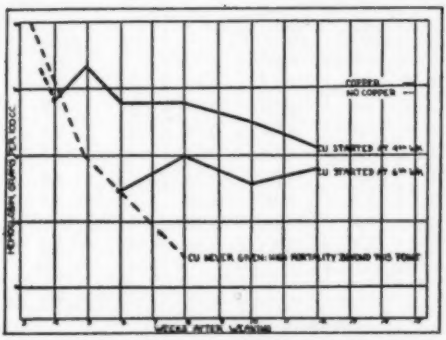

Fig. 1 Hemoglobin curves showing the effect of adding copper to the whole milk diet of anemic rats (average of ten males).

rise in hemoglobin occurred when the copper addition was made at the fourth week after weaning, when the hemoglobin level in these animals approximated 5 gm. per 100 cc. and also when the addition was made at the sixth week when the hemoglobin concentration has fallen to approximately 3.5 gm. per cent. Thus in both cases a body store of iron which became available for hemoglobin synthesis on the addition of copper was indicated, in spite of the low blood hemoglobin level characteristic of severe anemia.

These findings are in keeping with those of Schultze and Elvehjem ('33) referred to before, and substantiate their suggestion that "even severely anemic rats contain a small

amount of iron which may be used for hemoglobin building in the presence of adquate amounts of copper." Our attention has recently been called to the work of Hubbell ('36) who found that the feeding of copper to anemic rats (4 to 5 gm.

TABLE 1

Hemoglobin regeneration in anemic rats at different levels of iron intake. Comparison of hemoglobin gains in rats made anemic in the presence or absence of copper

AMOUNT OF Fe FED DAILY	RATS MADE ANEMIC IN ABSENCE OF COPPER				RATS MADE ANEMIC IN PRESENCE OF COPPER			
	Number of rats	Initial hemoglobin	6 weeks gain hemoglobin	± P.E.	Number of rats	Initial hemoglobin	6 weeks gain hemoglobin	± P.E.
	Males				Males			
mg.		*gm./100 cc.*	*gm./100 cc.*			*gm./100 cc.*	*gm./100 cc.*	
0.000	6	4.3	0.30		8	5.0	—0.60	0.20
0.014	14	4.0	1.10	0.18	10	5.5	0.50	0.11
0.050	20	4.0	3.50	0.14	12	5.6	1.40	0.21
0.071	5	3.9	4.30	0.24	13	4.8	2.80	0.28
0.100	14	4.0	5.50	0.14	13	4.8	3.00	0.28
0.150	11	4.0	8.20	0.21	13	4.9	5.40	0.36
0.200	12	3.8	9.10	0.19	10	4.1	5.70	0.29
0.250	12	3.5	10.40	0.18	8	4.0	6.90	0.40
0.300	7	4.1	10.10	0.29	10	3.9	7.90	0.23
0.400					1	3.9	9.60	
0.500					3	3.4	10.50	
	Females				Females			
0.000	12	6.0	0.10		12	6.0	—0.42	0.16
0.014	5	4.1	2.30	0.32	12	6.4	0.71	0.25
0.050	13	4.4	4.20	0.33	11	5.8	2.08	0.37
0.071	15	3.9	5.60	0.25	11	5.9	2.75	0.30
0.100	12	4.0	6.50	0.15	10	5.9	4.50	0.23
0.150	10	3.7	9.50	0.26	10	6.2	5.70	0.39
0.200	8	3.7	10.90	0.25	8	5.5	6.10	0.37
0.250	6	4.1	10.70	0.18	11	5.2	7.40	0.31
0.300	7	4.6	10.20	0.16	9	4.0	8.40	0.35
0.400					5	3.8	10.10	
0.500					2	3.8	10.70	

per cent) caused an increase in hemoglobin of from 0.4 to 1.5 gm. She states, however, that "these slight increases were within normal variability in hemoglobin and did not indicate any necessity for administration of copper during the preparation period to insure full exhaustion of iron reserves."

That the iron reserves in anemic rats whose hemoglobin level has fallen to a low level, though relatively small, does have an appreciable effect upon the subsequent response of these rats to iron supplementation is quite apparent from our experiments, however. Comparative hemoglobin responses to iron and copper supplementation of rats made anemic in the presence or absence of copper in the foreperiod are tabulated in table 1 and shown graphically in figure 2. At all levels of iron feeding, the rats which had not received copper in the anemia preparation period regenerated more

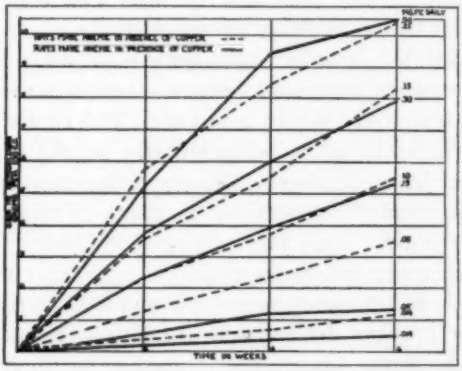

Fig. 2 Hemoglobin curves showing difference in response to iron supplementation between rats made anemic in the presence of copper and those given no copper in the anemia preparation period.

hemoglobin than those which had been made anemic on the whole milk diet supplemented with copper from the fourth week on.

For example, the copper-in-foreperiod rats given 0.05 mg. of iron as ferric chloride daily plus 0.05 mg. copper as copper sulfate gained only 1.4 gm. of hemoglobin per 100 cc. blood in the 6-week test period, whereas the rats receiving the same iron and copper supplements, but which were not made anemic in the presence of added copper showed an average hemoglobin regeneration of 3.5 gm. per cent in a test period of the same length.

Again, it may be seen that when rats have been made anemic in the presence of copper 0.3 mg. of iron as ferric chloride, adequately supplemented with copper and manganese, given daily did not raise the hemoglobin to the normal level in a 6-week test period.

Thus, in every case, the gains in hemoglobin of the anemic rats which had not received copper in their preparation period were abnormally large and therefore cannot be considered a true measure of their response to the iron supplementation. It would seem, therefore, that unless rats were made anemic in the presence of sufficient copper for the complete utilization of iron reserves, their use for measurement of available iron in foods may give erroneous results.

A difference in response between males and females as previously reported (Smith and Otis, '37 b) may still be observed, hemoglobin gains being greater in the female at the same level of iron. However, the sex difference observed is of smaller magnitude when copper is given in the last 2 weeks of the foreperiod. This fact strongly indicates that part, if not all, of this sex difference is due to a greater store of iron in the female of the same hemoglobin concentration, and consequently a greater difficulty in depletion, rather than to a difference between males and females in their ability to utilize the iron supplements. A longer period of copper feeding to insure complete absence of residual iron might be expected therefore to erase all sex differences in response to iron.

It is interesting to note that although the hemoglobin concentration in the females receiving copper in the foreperiod was higher at the beginning of the test period than that of the females receiving no copper, their response to iron and copper supplementation was much less. This fact gives further evidence for our belief that a low hemoglobin concentration cannot safely be used as criterion of the absence of residual iron, for the store was obviously less in the females receiving copper in spite of their higher hemoglobin concentrations.

CONCLUSIONS

Rats made severely anemic upon a whole milk diet according to the Elvehjem-Kemmerer technic contain residual iron which is converted into hemoglobin when copper alone is administered in adequate amounts. Animals which do not have their iron reserves depleted in the presence of copper regenerate much more hemoglobin in response to subsequent iron and copper supplementation than do rats which have been given sufficient copper in the preparation period for complete utilization of iron stores.

LITERATURE CITED

ELVEHJEM, C. A., AND A. N. KEMMERER 1931 An improved technique for the production of nutritional anemia in rats. J. Biol. Chem., vol. 93, pp. 189–196.

ELVEHJEM, C. A., AND W. C. SHERMAN 1932 The action of copper in iron metabolism. J. Biol. Chem., vol. 98, pp. 309–319.

HART, E. B., H. STEENBOCK, J. WADDELL AND C. A. ELVEHJEM 1928 Iron in nutrition. VII. Copper as a supplement to iron for hemoglobin building in the rat. J. Biol. Chem., vol. 77, pp. 797–812.

HUBBELL, H. J. 1936 An investigation of the influence of sex on the utilization of iron. Dissertation, Columbia University, pp. 1–45.

JOSEPH, H. W. 1932 Studies on iron metabolism and the influence of copper. J. Biol. Chem., vol. 96, pp. 559–571.

SCHULTZE, M. O., AND C. A. ELVEHJEM 1933 The relation of iron and copper to the reticulocyte response in anemic rats. J. Biol. Chem., vol. 102, pp. 357–371.

SMITH, M. C., AND L. OTIS 1937 a Hemoglobin regeneration in anemic rats in relation to iron intake. J. Nutrition, vol. 13, pp. 573–582.

———— 1937 b Sex variations in utilization of iron by anemic rats. Science, vol. 85, pp. 125–126.

ON THE IDENTITY OF THE GOLDBERGER AND UNDERHILL TYPES OF CANINE BLACKTONGUE. SECONDARY FUSO-SPIROCHETAL INFECTION IN EACH [1, 2]

DAVID T. SMITH, ELBERT L. PERSONS AND HAROLD I. HARVEY [3]

Departments of Bacteriology and Medicine, Duke University, Durham, North Carolina

ONE FIGURE

(Received for publication, April 7, 1937)

Chittenden and Underhill ('17) described a new experimental disease of dogs characterized by anorexia, weight loss and a rapidly progressive inflammatory process in the mucus membranes of the mouth. The condition was produced by feeding a simple diet, adequate in proteins and calories but deficient in vitamins A and D. In 1923, Goldberger and Wheeler ('28) produced a similar disease by feeding a different diet, deficient in 'pellagra preventive' factors but otherwise adequate for nutrition. This picture was identified with canine blacktongue, a disease frequently recognized by southern veterinarians, and was named 'experimental blacktongue.' It could be cured by the addition of yeast or red meat to the diet.

[1] This is no. 7 in a series of coordinated studies on pellagra in man and associated deficiency diseases of animals from the Departments of Physiology, Pathology, Bacteriology and Medicine of Duke University with the coperation of Dr. Y. Subbarow of the Department of Biochemistry of Harvard Medical School. Previous publications are listed in the 'Literature Cited' as Smith ('32), Ruffin and Smith ('34), Smith and Sprunt ('35), Dann ('36), Ruffin and Smith ('37) and Smith and Ruffin ('37).

[2] The expenses of this work were covered in part by a grant from the American Cyanamide Company of New York and the Lederle Laboratories, Pearl River, N. Y.

[3] Aided by the Henry Strong Denison Medical Foundation.

Underhill and Mendel ('28), recognizing that a similar experimental disease appears as a result of two different deficient diets, reported very extensive experiments. They were able to produce their disease on diets containing large amounts of red meat and yeast. No adequate explanation has ever been offered for the striking similarity of the syndromes resulting from such dissimilar diets.

Since both these syndromes were characterized by fetid breath and gangrene of the mouth, it occurred to us that this similarity might be due to an identical infection secondary to different nutritional deficiencies. Goldberger's experimental blacktongue and the Underhill syndrome have been produced in this laboratory and the type of secondary infection studied.

EXPERIMENTS WITH THE GOLDBERGER TYPE OF CANINE BLACKTONGUE

Three dogs were fed Goldberger's diet no. 268 (Goldberger and Wheeler, '28) without modification, and the clinical picture of blacktongue was produced, with death in 94 to 107 days. For further study Goldberger's diet no. 123[4] was modified by substituting Underhill and Mendel's more complete salt mixture ('28) for the sodium chloride and calcium carbonate, yellow corn meal for the white corn meal, and by doubling the amount of cod liver oil. The commercial casein (Merck) was leached with acidulated water and extracted with 95% alcohol.[5] The diet used consisted of yellow corn meal, 400 gm., coarsley ground California black-eyed

[4] This diet was recommended by Dr. W. H. Sebrell, U.S.P.H.S., P.A. Surgeon, in charge of F. S. Nutrition, Washington, D. C.

[5] Method: 25 pounds of commercial casein are treated with 30 liters of distilled water acidified with 0.2% glacial acetic acid (60 cc.). The mixture is hand stirred frequently over a 48-hour period with constant agitation maintained by a stream of air led to the bottom of the 12½-gallon container. The acidified water is then poured off through a multilayered gauze screen. The water is absorbed, to promote drying, and further extraction carried on during the next 48 hours by adding 20 liters of 95% alcohol, with frequent stirring. The alcohol is decanted as above. The casein is then spread thinly over a large surface area to dry. Drying may be speeded up by currents of air as generated by electric fans.

peas (Vigna sinensis) 50 gm., casein 60 gm., sucrose 32 gm., cottonseed oil 30 gm., cod liver oil 30 gm., and salt mixture 12 gm. This supplied a total of 2535 calories. The cornmeal, cow peas and salt mixture were diluted with 3 volumes of water and cooked for 2 hours in an open steam cooker. After cooking, the other ingredients were added and thoroughly mixed. This food was fed ad libitum and a minimum of 125 calories per kilogram of body weight was offered each day.

Using this diet, canine blacktongue was produced ninety-six times in fifty-three different adult dogs of various breeds and both sexes. In the acute attack, smears and dark field preparations from the mouth lesions showed the constant presence of large numbers of fuso-spirochetal organisms. These organisms may be found in small numbers about the teeth in normal dogs (Smith, '32). In our experience, they always increase enormously in numbers during the acute stage of blacktongue and almost disappear after recovery induced by dietary treatment (Harvey et al., unpublished data) only to reappear with the next attack.

Material for culture and animal inoculation was obtained from the mouth of a dog in a severe attack of blacktongue. Saliva, containing mucus and small pieces of necrotic tissue, was aspirated from the dog's mouth with a sterile pipette. Smears of the material were stained by Gram's method, gentian violet, and Fontana's silver nitrate and preparations were studied with the dark field apparatus. The following organisms were tentatively identified: Treponema microdentium, Treponema macrodentium, Treponema vincenti, Treponema buccale, Leptospira trimerodonta, Spirillum sputigenum, fusiform bacilli, vibrios, and gram positive cocci, singly, in pairs, and chains (fig. 1). No spore bearing organisms were found by smear or culture. Two guinea pigs were inoculated in the groin with 1 cc. and two with 2 cc. of the fresh material from the dog's mouth. After 6 to 10 days all four animals developed abscesses in the groin which apparently contained all the original organisms except the Leptospiras. Anaerobic cultures from these abscesses were

also negative for spore bearing organisms. Similar abscesses were produced in the groins of other guinea pigs by inoculating 0.25 cc. to 0.5 cc. of the undiluted pus. Pus obtained from the third serial transfer of the infection in guinea pigs was introduced into the lungs of four normal dogs through a bronchoscope.[6] One dog escaped infection, one died in 48

Fig. 1 Drawing (× 1000) of a dark field preparation made from the saliva of a dog with the Goldberger type of blacktongue. The small tightly coiled spirochetes in the left upper quadrant of the drawing are morphologically identical with Treponema microdentium (Noguchi). The larger tightly coiled spirochetes at the top of the drawing resemble Treponema macrodentium (Noguchi). The thin irregularly coiled spirochetes in the center are Treponema Vincenti (Blanchard). The thick spirochetes showing double centers near the bottom of the drawing are apparently Treponema buccale (Luhe) (Dobell). In the upper right segment are leptospiras which may be Leptospira trimerodonta (Hoffmann). Scattered about in the field are fusiform bacilli which may be recognized by their characteristic shape. The small comma-shaped organisms are vibrios and the larger curved organisms are spirilla resembling Spirillum sputigenum (Miller). Cocci, singly, in pairs, and short chains are always present.

The leptospiras are not always found but the other organisms are constantly present in varying proportions in this type of infection.

[6] We wish to thank Dr. W. W. Eagle, of the Department of Surgery, in charge of Otolaryngology, for his assistance in introducing this material into the dogs' bronchi.

hours with an extensive pneumonitis, the other two died in 8 to 10 days with pulmonary abscesses of the fuso-spirochetal type. The organisms found in all these lesions were morphologically identical with those present in noma, Vincent's angina, and pyorrhea in man.

EXPERIMENTS WITH THE UNDERHILL SYNDROME IN DOGS

The diet used by Underhill and Mendel in their experiment no. 18 ('28) was fed to four dogs. The diet consisted of raw beef, cracker meal, cottonseed oil, yeast and Underhill and Mendel's salt mixture. In two additional dogs a part of the cottonseed oil was replaced by cod liver oil to supply 3.3 gm.[7] per kilogram of body weight per day. The animals which did not receive cod liver oil developed the typical syndrome of weight loss and progressive buccal necrosis and died after 54, 66, 81 and 133 days respectively. The two dogs which received cod liver oil remained well and were returned to stock after 152 days, which was 36 days longer than the maximum time required by Underhill and Mendel to produce the disease. These results indicate that the cod liver oil, used by us, contains a preventive factor.

The possibility that a deficiency of either vitamins D or C, or both, might contribute to the development of the syndrome prompted us to repeat the experiment, supplying 600 units of vitamin D, in the form of viosterol, and 1 mg. of ascorbic acid per kilogram of body weight per day. Four animals developed the characteristic syndrome on the Underhill and Mendel diet supplemented by vitamins C and D but not by cod liver oil, and died after 30, 76, 129 and 130 days. Two control dogs were fed as in the previous experiment receiving 3.3 gm. of cod liver oil per kilogram per day but no viosterol or ascorbic acid. One lived 137 days without the appearance of any mouth lesions and died of hook worm infestation, the other remained well for 156 days and was returned to stock.

[7] Mead-Johnson cod liver oil. One gram contained 1750 U.S.P. units of vitamin A and 175 units of vitamin D.

These experiments indicate that the Underhill syndrome can be prevented by some factor in the cod liver oil, and that this factor is not vitamin D. Underhill and Mendel ('28) could prevent or cure the disease with yellow butter, carrots or carotin, but not with cod liver oil. It seems probable that vitamin A is the significant factor and that the cod liver oil which they used contained fewer vitamin A units than the product used at present.

The use of these entirely different deficient diets in the same laboratory has given us convincing evidence that the clinical pictures of Goldberger's experimental blacktongue and the Underhill syndrome are identical. The bacteriologic findings were also identical. Smears and dark field preparations showed the presence of the same variety of organisms previously described (fig. 1). Material from the mouth of one of the dogs was inoculated into the groin of a guinea pig and the animal developed a typical fuso-spirochetal abscess after 4 days. The infection was successfully passed through four series of guinea pigs. Aerobic and anaerobic cultures were negative for pyocyaneus and gas gangrene organisms.

DISCUSSION

Fuso-spirochetal organisms were demonstrated in enormous numbers in the oral lesions occurring in dogs with each of the two types of dietary deficiency studied. There has been lengthy discussion as to the pathogenicity of the fuso-spirochetal group of organisms. The literature on this subject has been extensively reviewed elsewhere (Smith, '32), but the experiments reported here show that the group of organisms obtained from the mouths of the sick dogs is capable of producing severe infection in normal animals when the inoculation is made with minimal trauma. There is evidence (Smith, '30; Proske and Sayers, '34; Proske, '34) of a true symbiosis of these organisms as the causative factor of Vincent's angina, pyorrhea and fuso-spirochetal disease of the lungs.

Since Goldberger's experimental blacktongue is curable by yeast and meat, but not by vitamin A (Goldberger and Wheeler, '28) while the Underhill diet contains yeast and meat, the discovery of identical pathogenic groups of microorganisms in the very similar buccal lesions must be regarded as significant. In this connection it is important that Wallace, Wallace and Robertson ('33) reported the production of the same type of canine mouth lesions on diets adequate for nutrition. The lesions appeared after daily intravenous injections of one-fourth the lethal dose of scillaren B, a squill glucoside. 'Typical smears of Vincent's angina' are reported but no mention is made of organisms other than fusiform bacilli and spirochetes. Inoculation of pus from the lesions into the gums of normal dogs failed to reproduce the disease. The gross and microscopic pathology was described as very similar to that in human cases of Vincent's angina.

Miller and Rhoads ('35) observed the presence of fuso-spirochetal organisms in the oral lesions of dogs developing blacktongue on the Goldberger diet. They were unable to infect normal dogs by inoculating masses of these organisms into and under the labial mucus membrane. In our experiment we succeeded in producing infection in guinea pigs by inoculating large amounts of the material into the loose areolar tissue in the groin where the organisms find a favorable place for development.

It apears, then, that organisms of the fuso-spirochetal group which are normally present in the dog's mouth may, in at least one type of drug intoxication and two different types of nutritional disturbance, produce a necrotizing infectious process similar to that observed in spontaneous canine blacktongue. Recognition of the fact that entirely different factors may lower tissue resistance, and result in infection with the same group of organisms, should not vitiate the results of adequately controlled nutritional studies in which the secondary lesions are used as an indication of nutritional status.

At the same time, this concept furnishes a long-sought explanation for the similarity of the Underhill and the Goldberger syndromes.

These findings may have some bearing on problems of human nutrition. Fuso-spirochetal organisms are found almost invariably in the oral, and frequently in the vaginal, lesions of patients with acute pellagra. This association, well known in the South, has also been observed in Cleveland by Blankenhorn and Spies ('36); Spies, ('35) in alcoholic pellagrins. Sambon ('19) and Greenbaum ('27) have found the same organisms in the skin lesions of pellagra. Fuso-spirochetal infection in the mouth of a pellagrin disappears after treatment with materials potent in the pellagra-preventive factor. The Goldberger type of blacktongue is cured by the same materials (Harvey et al., unpublished data), indicating that this is the type of blacktongue which is analogous to pellagra.

CONCLUSIONS

1. The Underhill syndrome appears in dogs receiving meat and yeast and is prevented by adequate amounts of cod liver oil, whereas the Goldberger type of blacktongue appears in dogs receiving adequate amounts of cod liver oil, and is prevented by meat or yeast.

2. The Underhill syndrome in dogs is clinically and bacteriologically identical with Goldberger's experimental canine blacktongue.

3. The oral lesions in both the Goldberger and Underhill types of canine blacktongue are the result of infection with the fuso-spirochetal group of organisms secondary to lowered tissue resistance.

4. Dogs on the Goldberger diet may be used with confidence in assaying the pellagra curative value of various substances, because the appearance of secondary fuso-spirochetal infection is a reliable indicator of the nutritional status.

LITERATURE CITED

BLANKENHORN, M. A., AND T. D. SPIES 1936 The oral complications of chronic alcoholism. J. Am. Med. Assoc., vol. 107, p. 641.

CHITTENDEN, R. H., AND F. P. UNDERHILL 1917 The production in dogs of a pathological condition which closely resembles human pellagra. Am. J. Phys., vol. 44, pp. 13–66.

DANN, W. J. 1936 The vitamin G complex; the non-identity of rat dermatitis due to vitamin B_6 deficiency and the dermatitis of human pellagra. J. Nutrition, vol. 11, pp. 451–462.

GOLDBERGER, JOSEPH, AND G. A. WHEELER 1928 Experimental blacktongue of dogs and its relation to pellagra. U. S. Pub. Health Rep., vol. 43, pp. 172–217.

GREENBAUM, S. S. 1927 Fusospirillary dermatitis; its occurrence in acute pellagra. Arch. Dermat. and Syph., vol. 15, pp. 678–684.

HARVEY, H. I., D. T. SMITH, E. L. PERSONS AND M. V. BURNS Unpublished data.

MILLER, D. K., AND C. P. RHOADS 1935 Experimental production in dogs of acute stomatitis associated with leucopenia and maturation defect of myeloid elements of bone marrow. J. Exp. Med., vol. 61, pp. 173–182.

PROSKE, H. O. 1934 Pulmonary infection in pneumocomosis; fuso-spirochetal infection. Experiments in guinea pigs. U. S. Pub. Health Rep., vol. 49, pp. 1212–1217.

PROSKE, H. O., AND R. R. SAYERS 1934 Pulmonary infection in pneumonocomosis; bacteriologic and experimental study. U. S. Pub. Health Rep., vol. 49, pp. 839–858.

RUFFIN, J. M., AND D. T. SMITH 1934 The treatment of pellagra with certain preparations of liver. Am. J. Med. Sci., vol. 187, pp. 512–521.

———— 1937 A clinical evaluation of the potency of various extracts of liver in the treatment of pellagra. So. Med. J., vol. 30, pp. 4–14.

SAMBON 1919 (Quoted by Castellani) Castellani and Chalmers 'Manual of Tropical Medicine,' 3rd ed. London, p. 1715.

SMITH, D. T. 1930 Fuso-spirochaetal disease of the lungs produced with cultures from Vincent's angina. J. Infect. Dis., vol. 46, p. 303.

———— 1932 Oral spirochetes and related organisms in fuso-spirochetal disease. Williams and Wilkins Company, Baltimore.

SMITH, D. T., AND J. M. RUFFIN 1937 Effect of sunlight on the clinical manifestations of pellagra. Arch. Int. Med., vol. 59, pp. 631.

SMITH, SUSAN G. 1932 A new symptom complex in vitamin G deficiency in rats. Proc. Soc. Exp. Biol. and Med., vol. 30, pp. 198–200.

SMITH, SUSAN G., AND D. H. SPRUNT 1935 Pathological skin changes in the tail of the albino rat on a diet deficient in vitamin G. J. Nutrition, vol. 10, pp. 481–492.

SPIES, T. D. 1935 The treatment of pellagra. J. Am. Med. Assoc., vol. 104, p. 1377.

UNDERHILL, F. P., AND L. B. MENDEL 1928 Pellagra-like canine deficiency disease possibly related to blacktongue. Am. J. Phys., vol. 83, pp. 589.

WALLACE, H., E. W. WALLACE AND O. H. ROBERTSON 1933 The production of experimental Plaut-Vincent's angina in the dog. J. Clin. Invest., vol. 12, p. 909.

IMMATURITY OF THE ORGANISM AS A FACTOR DETERMINING THE FAVORABLE INFLUENCE OF LACTOSE ON THE UTILIZATION OF CALCIUM AND PHOSPHORUS

ROWLAND B. FRENCH[1] AND GEORGE R. COWGILL
Laboratory of Physiological Chemistry, Yale University, New Haven

TWO FIGURES

(Received for publication April 3, 1937)

The literature concerning the influence of lactose on the utilization of calcium and phosphorus is full of contradictions which as yet have not been explained. Some of these studies have been made as part of an investigation of metabolism as affected by the parathyroid glands, others in relation to growth, and still others in connection with the effect of diet on intestinal flora.

From the work of Dragstedt and Peacock ('23) and Inouye ('24), it appears that lactose, in contrast to sucrose, dextrin and glucose, has the remarkable property of preventing tetany in the thyro-parathyroidectomized dog. Inasmuch as tetany is characterized by a fall in the level of blood calcium and a retention of phosphorus, it is reasonable to believe that lactose in some way influences favorably the body's metabolism of calcium.

Lactose feeding has been shown to bring about changes in the pH of the contents of the whole intestinal tract below the duodenum (Robinson and Duncan, '31) and to favor the development of an acidophilic flora (Rettger and Cheplin, '23). The bacteriological studies of Cannon and McNease ('23) and Cruickshank ('28) might be cited as favoring the view that any effect of lactose on calcium metabolism is a specific one and not the result merely of an increased solubility of calcium

[1] Now located at Agricultural Experiment Station, Gainesville, Florida.

salts in the more acid medium. This view receives further support by the experiments of Kline, Keenan, Elvehjem and Hart ('32) with young chicks subsisting on a rachitic ration.

It is pertinent to point out that the negative results of Gross ('27) were obtained on mature dogs, whereas the positive findings reported by Bergeim ('26) for rats, Robinson, Huffman and Mason ('29) for calves, and Kline, Keenan, Elvehjem and Hart ('32) for chicks were obtained from immature organisms. Is it possible that immaturity in some way plays a definite role?

In view of the conflicting results cited, it seems obvious that the degree of influence of lactose on calcium and phosphorus utilization, if it exists, must be small. One might expect the slight effects of added lactose to be magnified when the diet contains only very small amounts of either calcium or phosphorus. In the experiments here reported the study of the effects of lactose was made under conditions where the amounts and ratios to each other of calcium and phosphorus have been carefully controlled.

In the course of this work, in which balance studies were made on three female dogs, definitely positive effects of lactose on calcium and phosphorus retention were noticed with two of the animals and no effect whatsoever with the third dog. In seeking for an explanation of this result, we were led to consider the obvious fact that the two animals yielding favorable results were much younger and immature in contrast to the third dog which was quite old. The previous history of these animals was unknown. Therefore we are unable to describe in any really precise terms the 'degree of immaturity' characterizing these particular young dogs.

Further experiments with rats likewise gave some data at variance with others. Consideration of the 'degree of immaturity' of the animals yielding the respective data, in these cases known by the age and body weights of the rats, again suggested very definitely that there is a factor related in some way to the effect of lactose on calcium and phosphorus utilization. In reporting these experiments, therefore, we have taken this possible relationship as the theme of this paper.

PART I

BALANCE STUDIES IN DOGS

Plan of experiment

The plan of investigation followed in part I of this study consisted of the determination of calcium and phosphorus balances in consecutive periods differing in only one respect, namely, the presence or absence of lactose in the diet. In comparable experimental periods diets were used that were low in either calcium or phosphorus. Other factors taken into account were the possible influence of the protein in the diet and the age of the dog. Either dried coagulated egg albumen or the phospho-protein casein were used as the source of protein in comparable periods. One mature dog and two immature animals yielded the data reported in part I of this paper.

In the experimental routine the dogs were fed the particular diet in question for a period of 5 days to allow some adjustment to the new dietary conditions; this was followed by a 5-day period during which collections were made and the balances determined. At the beginning and the end of each period the dogs were fed carmine to mark the stools and catheterized to secure complete emptying of the urinary bladder. The dog's food for each period was weighed out at the beginning and a sample taken at that time for chemical analysis. The urine was collected daily, measured and made up into a composite sample which was analyzed. The treatment of the feces was similar except that the composite sample was dried, weighed, ground and saved for analysis in a sealed bottle. Blood analyses for calcium and phosphorus were performed in all experimental periods.

Methods of analysis

For the calcium analyses of food, feces and urine, McCrudden's ('10, '11) method was used. Blood calcium was determined by the Clark-Collip ('25) modification of the Kramer-Tisdall method. Neumann's ('02) technic was employed for the phosphorus analyses performed on the food, feces and urine, and the blood plasma phosphorus was determined by Briggs' ('24) modification of the Bell and Doisy procedure.

Composition of diets

The compositions of the diets and salt mixtures used in this study are given in tables 1 and 2. In constructing the experimental rations the kilo-unit plan described by Cowgill ('23) was followed.

The duration of each balance period was 5 days with a 5-day interval between them to allow an adjustment to the new dietary regime. The plan of experiment involves the assumption that the utilization of calcium and phosphorus over a period of 15 days is constant except for the reaction of the animal toward the one change made in the diet, namely, the substitution of lactose for some of the sucrose.

RESULTS

Adequacy of technic. Bauer, Albright and Aub ('29) showed that it is possible under uniform conditions to obtain duplicate negative calcium balances on human beings. On the same person consecutive balance experiments checked satisfactorily but there were considerable variations between different subjects on the same diet that were not readily explained. Our first experiment was designed to yield confirmatory data on this point and to assure us that our technic was adequate for the problem being studied. The result was most satisfactory and readily confirmed in later experiments. As an example of these data we present the results for dog 2, period 1, shown in table 3. It will be noticed that two separate experiments with the diet of 'period 1' were made with dog 2. The results with respect to the calcium balances are almost identical; the data pertaining to phosphorus do not agree so well. In explanation of this difference we can only offer the consideration that phosphorus seems to play a more varied role in metabolism and therefore may not be as readily controlled as calcium.

Effect of lactose during a low-calcium dietary regime. In all of the experiments the diets were made up with the carbohydrate constituting 40% of the mixture. When lactose was added it was substituted for one-half of the sucrose. In

TABLE 1

Composition of diets

Ration 1: For low-calcium regime

INGREDIENT	GRAMS	CALORIES	PER CENT
Egg albumen (coagulated), 11.91% N	5.78	17.2	32.0
Lard	2.80	25.2	15.5
Butter fat	1.20	10.8	6.6
Sucrose	7.25	29.0	40.2
Cellu flour	0.6		3.3
Salt mixture no. 1	0.43		2.4
Total—kilo unit	18.06	82.2	100.0

Ration 2: low Ca plus lactose; same as ration 1 except that lactose is substituted for one-half the sucrose.

Ration 3: low P; same as ration 1 except that salt mixture no. 2 is used in place of salt mixture no. 1.

Ration 4: low P plus lactose; same as ration 2 except that salt mixture no. 2 is used in place of salt mixture no. 1.

Ration 5: low Ca; same as ration 1 except that 5.46 gm. of casein containing 12.6% N replaces the egg albumen.

Ration 6: low Ca plus lactose; same as ration 5 except that lactose is substituted for one-half the sucrose.

TABLE 2

Composition of salt mixtures

COMPONENTS	OSBORNE-MENDEL ('19)	NO. 1	NO. 2
	gm.	gm.	gm.
Calcium carbonate	134.8	....	134.8
Magnesium carbonate	24.2	24.2	24.2
Sodium carbonate	34.2	34.2	34.2
Potassium carbonate	141.3	141.3	141.3
Potassium iodide	0.02	0.02	0.02
Phosphoric acid	103.2	103.2	....
Hydrochloric acid	53.4	53.4	53.4
Manganese sulfate	0.079	0.079	0.079
Sulfuric acid	9.2	9.2	9.2
Citric acid	111.1	....	....
Ferric citrate	6.34	6.34	6.34
Sodium fluoride	0.248	0.248	0.248
Potassium alum	0.0245	0.0245	0.0245
Total	618.1115	372.2115	403.8115
Amounts used in grams:			
Per 100 gm. of food	4	2.41	2.61
Per kilo unit	0.74	0.43	0.47

TABLE 3

Calcium and phosphorus balances in dogs on diets with and without lactose

BASIC DIETARY REGIME	DOG NO.	DIET NO.[1] (L = LACTOSE)	CALCIUM PER DAY (AVERAGE)				PHOSPHORUS PER DAY (AVERAGE)				BLOOD (PER 100 CC.)	
			Intake	Output		Balance	Intake	Output		Balance	Ca	P
				Feces	Urine			Feces	Urine			
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	mg.	mg.
Low calcium	1 Immature	5: without L	0.101	0.170	0.008	−0.077	1.119	0.158	0.974	−0.013	12.1	5.9
		6: with L	0.121	0.110	0.017	−0.006	1.113	0.126	0.850	+0.137	12.8	5.9
	2 Immature	1: without L	0.061	0.161	0.026	−0.126	0.540	0.084	0.517	−0.061	12.1	5.6
		1: without L	0.066	0.168	0.026	−0.128	0.547	0.090	0.498	−0.041	11.6	6.7
		2: with L	0.061	0.109	0.010	−0.058	0.556	0.065	0.480	+0.011	11.8	4.7
	2 Immature	5: without L	0.117	0.095	0.004	+0.018	1.291	0.083	1.127	+0.081	12.2	5.7
		6: with L	0.141	0.038	0.015	+0.088	1.285	0.088	1.033	+0.164	12.3	6.1
	3 Mature	5: without L	0.069	0.063	0.004	+0.002	0.791	0.240	0.536	+0.015	10.8	4.7
		6: with L	0.082	0.079	0.003	0.000	0.788	0.457	0.322	+0.009	11.1	4.8
Low phosphorus	1 Immature	3: without L	0.565	0.586	0.019	−0.040	0.278	0.134	0.194	−0.050	12.0	4.8
		4: with L	0.622	0.394[2]		+0.228	0.268	0.190[2]		+0.078	12.3	6.0
	2 Immature	3: without L	0.639	0.509	0.010	+0.120	0.287	0.087	0.261	−0.061	12.1	6.3
		4: with L	0.705	0.520[2]		+0.185	0.276	0.083[2]		+0.193	13.6	6.1
	3 Mature	3: without L	0.400	0.405	0.006	−0.011	0.153	0.103	0.061	−0.011	10.6	4.8
		4: with L	0.441	0.459[2]		−0.018	0.146	0.164[2]		−0.018	11.7	4.0

[1] For detailed composition of these diets see table 1.

[2] Total for feces and urine; could not collect these excreta separately. See text for discussion of laxative effect of diet 4.

Inouye's ('24) study of the value of lactose in preventing and curing parathyroid tetany, the ratio of the amount of casein in the diet to the lactose fed was found to be very important. Various diets which failed to prevent tetany had casein/lactose values as high as 8; rations which were borderline had values ranging from 1 to 4; diets which definitely protected against the development of tetany had values as low as from 0.08 to 1. In view of these data it was our expectation that any influence of lactose on calcium and phosphorus balances would be most marked when the ratio of protein to lactose in the experimental diets approximated the value of 1. Actually the ratio for our rations proved to be 1.3. A summary of the data is presented in table 3.

Examination of table 3 reveals that in the case of the two immature dogs on a low-calcium regime, substitution of lactose for one-half of the sucrose resulted in marked improvement in both the calcium and the phosphorus balances. In the case of dog 1, the use of lactose was associated with a change of the calcium balance from a daily loss of 0.077 gm. to approximate equilibrium and the phosphorus balance was altered from a moderately negative condition to one distinctly positive. Dog 2 was used in two separate experiments, one with diets 1 and 2, in which egg albumen was the protein used, and the other with diets 5 and 6 which contained casein as the protein; in the latter case the balances without lactose were positive, and the addition of this carbohydrate was associated with increased retention of both calcium and phosphorus. In contrast to these results obtained with immature dogs one sees the data for the mature animal, dog 3, indicating insignificant changes in the calcium and phosphorus balances, the larger difference in the case of the phosphorus being within the range of differences noted in duplicate experiments (dog 2, period 1).

The changes in the utilization of calcium associated with the presence of lactose are large enough to suggest that this carbohydrate acts in some way to diminish the rate of excretion of calcium. The increase in the amount of Ca utilized by dog 2, when changed from diet 1 to diet 2, is slightly greater than the total quantity of the element furnished in the ration.

Effect of lactose during a low-phosphorus dietary regime. For this phase of the investigation rations 3 and 4, low in phosphorus content, were devised (table 1), the salt mixtures being altered to meet the requirements of the experiment. Examination of the data in table 3 reveals that in immature dogs the use of lactose together with a dietary low in content of phosphorus results in improvement of both the calcium and the phosphorus balances. This result is again in marked contrast to that characterizing the mature animal.

Egg albumen was used as one of the proteins of the diets because the plan of experiment required use of a diet low in phosphorus content. Being available in quantity, and lacking both calcium and phosphorus, the two variables of greatest interest in these experiments, this protein was selected.

Bateman ('16) has shown that ingestion of appreciable quantities of raw egg-white results in diarrhea in both animals and man. For our experiments, therefore, the egg albumen (commercial scales) was coagulated by heat, dried and ground before being incorporated in the test diets. When the egg albumen rations were fed, the resulting freshly voided feces had a greenish color which rapidly turned black on standing; this necessitated the use of carmine rather than charcoal to differentiate the stools.

It is an interesting fact that the favorable influence of lactose on the utilization of calcium and phosphorus was observed whether the protein was coagulated egg albumen or the phospho-protein casein.

Lactose had a definitely laxative effect in all the dogs. Egg albumen acted similarly. In the experiments with diet 4 (low phosphorus plus lactose) the cumulative effect of lactose and the protein on the consistency of the feces was such that a separation from the urine could not be made by the method used. Under these conditions, nevertheless, the favorable influence of lactose on the utilization of calcium and phosphorus was observed, from which it appears that even when considerable laxation prevails lactose still has the property of increasing the availability of calcium and phosphorus to the immature animal.

The fact that no such effect was observed in our mature dog suggests that the age of the animal, or its degree of maturity, is significant in this connection. It is possible that the age factor is significant because in the young animal there is a more active bone metabolism associated with rapid growth, whereas in the mature organism there is no necessity for utilization of calcium and phosphorus beyond the requirements for maintenance except under particular conditions such as pregnancy and great activity of the mammary glands during lactation. It appears that the production of cataract in rats by high-lactose diets is likewise related in some way to the age of the animal, young rats being definitely more susceptible in this respect (Mitchell and Dodge, '35).

PART II

EXPERIMENTS WITH RATS

Does lactose act to favor absorption or to diminish the excretion of calcium and phosphorus? Examination of the data in part I for distribution of excreted calcium and phosphorus between the feces and urine does not reveal a definite answer to this question. Bergeim ('26) has shown that in both normal and rachitic rats there is good absorption of calcium from the small intestine, the secretion of phosphate into the gut favoring such absorption. Bergeim's method consists essentially in adding to the ration a substance which is practically unabsorbable, ferric oxide, for example, and then studying the ratio of this substance to calcium and to phosphorus in different parts of the gastro-intestinal tract. In this connection it is pertinent to point out that the experiments, in which Bergeim demonstrated the favorable effect of lactose on the retention of calcium and phosphorus, were conducted on young rats. Using Bergeim's method and a diet low in calcium content one might expect the absorption of calcium in the upper part of the intestinal tract to be fairly complete. If this assumption is correct, then differences in increases of calcium concentration in lower parts of the tract should be due to changes in rates of elimination. It was therefore decided to

test this idea on rats using Bergeim's technic. These experiments are described in this section. In the course of this phase of the study additional evidence was obtained indicating the importance of degree of maturity of the organism in determining the effect of lactose on the utilization of calcium and phosphorus.

Analytical methods

The methods for determination of calcium and phosphorus were the same as those employed in the experiments described in part I. Phosphorus was determined colorimetrically because the quantity of iron in the samples was great enough to interfere seriously with the usual molybdate procedure. In the first experiments described below iron was determined by the simple method recommended by Bergeim ('26), but owing to the variable results obtained, the colorimetric thiocyanate technic as improved by Marriott and Wolf ('05–'06) was substituted and found to yield satisfactory duplicate results.

Preliminary experiments with a low-calcium diet

These trials of the Bergeim technic were performed on ten female rats distributed between two groups, one receiving diet 5 (low-calcium) and the other group diet 6, which was the same as diet 5 except that lactose was present to the extent of 20% replacing an equivalent amount of sucrose. Failure to secure any clear-cut results in these experiments led to a search for an explanation. To eliminate the possibility that the Bergeim method for determination of iron was inadequate under our conditions of study, the colorimetric thiocyanate method as improved by Marriott and Wolf ('05–'06) was substituted for the remaining experiments. There also occurred to us the possibility that significant but unappreciated quantities of calcium were being poured into the gastro-intestinal tract sufficient to off-set any effect that feeding a low-calcium diet might have. Therefore data on the amounts of calcium present in various secretions of the alimentary tract were sought in an attempt to weigh this possibility. From the data

reported by Austin and Mathews ('27) and Walch and Ivy ('27–'28) it appears that the concentrations of calcium in the saliva, gastric juice, pancreatic juice, bile and secretions of the lower ileum of the dog approximate those found in the blood, ranging from as low as 6 mg. per 100 cc. for gastric juice to as high as 11 for gall bladder bile. Obviously, when the amount of calcium contained in the food is very low, the quantities of this element contributed by the alimentary secretions assume great importance. Therefore we are justified as assuming as correct the view that the advantages one might expect to gain in using a low-calcium diet in this type of experiment are nullified by the appreciable amounts of calcium poured into the gastro-intestinal tract in the course of normal digestive processes. For this reason we did not repeat this phase of the study.

Experiments with complete diets containing varying amounts of lactose

Six groups of rats, two females in each, were used for this phase of the study. The animals were classified by weight and then fed complete diets containing levels of lactose ranging from 0 to 60%. Six rations were thus tested. The feeding was continued for a week, and then a 3-day collection of feces was made, specimens soiled with urine being discarded. The feces were dried, ground and analyzed for iron, calcium and phosphorus, as was the food. The ratios of iron to calcium and to phosphorus of both the food and the feces were then computed and compared. The results are presented graphically in figure 1.

In interpreting figure 1, it should be borne in mind that the differences between the heights of the columns in any given group are the significant features to be studied. In the upper row of columns are presented the ratios of calcium/iron and phosphorus/iron resulting from the analyses of the diets used. The middle row gives the calcium/iron ratios yielded by each of the two animals on the experiment; in the lower row are shown similar data referring to the phosphorus/iron ratio.

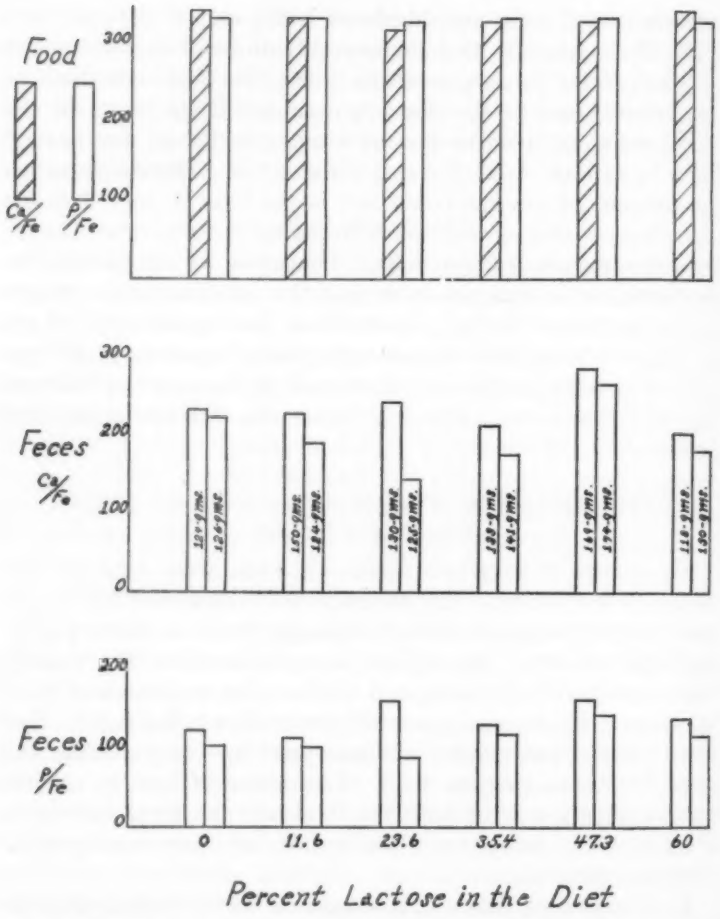

Fig. 1 The effect of varying amounts of lactose in the diet of rats on the ratios of calcium and phosphorus to iron in the feces studied by the Bergeim ('26) technic. The results presented in the two lower rows of columns—data pertaining to the feces—were obtained with two female rats for each experimental diet. Then body weights of the animals are given in the respective columns of the middle row. It will be noticed that the columns in the middle row, Ca/Fe, for the diet containing 47.3% of lactose are the highest, indicating the smallest utilization of calcium; these particular rats were the heaviest of the entire group and, by reference to figure 2, had passed the period of most rapid rate of growth.

The variations observed in the columns of the two lower rows are worthy of comment. In arriving at the ratio values for the diets, it is apparent that the additive errors of two chemical determinations are involved; furthermore, the determinations represented by the upper row of columns were made on six different rations. The fact that only very slight differences are shown in the columns of the upper row therefore indicates that the cause of variation in results summarized in the two lower rows of figure 1 must be sought elsewhere than in error of chemical analyses. Evidently they are due to biological factors difficult to control; that appreciable variability of results is common in work of this type appears evident also from the data of Bauer and associates ('29) who made very careful balance studies on human subjects and yet, in many instances, observed variations that could not be explained.

Examination of the columns in the middle row reveals that the two animals receiving the diet with 47.3% lactose showed the least utilization of calcium of all the groups, and the degree of utilization in this case was quite small. These two animals were the heaviest of the entire group, weighing 169 and 174 gm., respectively. It will be recalled that in the experiments on dogs, described in part I of this paper, the mature animal failed to show any increase in utilization of calcium and phosphorus when lactose was fed. We therefore considered the possibility that the degree of maturity of the rats used in these experiments was playing a role.

All of the rats were females obtained from four different litters; in age they ranged from 67 to 71 days at the time the experiments were begun. The weights of the animals, corresponding to the time when the data yielding the ratios indicated in figure 1 were obtained, ranged from 118 to 174 gm. These weights are indicated by the figures on the respective columns. It will be noticed that the two rats which showed the least utilization of calcium weighed the most, namely, 169 and 174 gm.

The question then presented itself: When may a female rat be said to have reached maturity or, as far as the problem of the moment was concerned, at what level of body weight (or age) is there a marked reduction in rate of growth, and with this a corresponding decrease in the demands of the body for a substance like calcium?

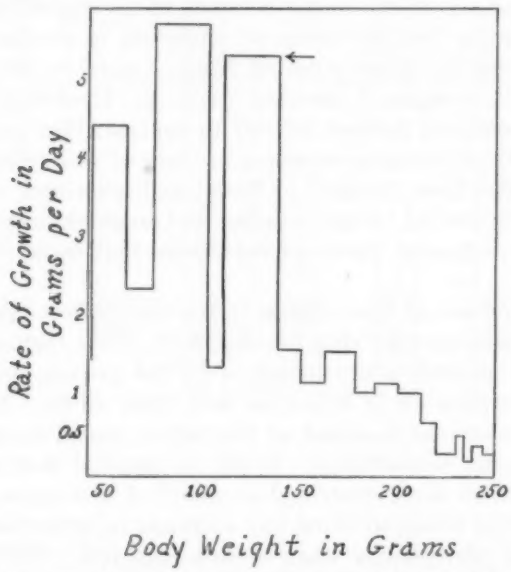

Fig. 2 This plot, based on the data of Smith and Bing ('28) for female rats, indicates that when a body weight of about 137 gm. has been attained, there is a marked decrease in the rate of growth referred to body weight. The variations shown on the graph previous to the arrow are not readily explained.

Smith and Bing ('28) have published data on the growth of female rats in this colony. Using their data we have plotted average daily gain in weight against average body weight for the same period. The result of such a plot is shown in figure 2.

From figure 2 it appears that when a female rat of this colony has attained the weight of about 137 gm. (see arrow), an average figure having a standard deviation of 12.8 and a coefficient of variation of 9.3%, that rat may be said to have

passed the period of most rapid growth, and therefore 'matured' in this sense. It is obvious that such a result as we have just cited may very well be conditioned by numerous factors operating in the colony of this laboratory.

The two animals that were definitely larger than the limit suggested by figure 2 weighed 169 and 174 gm., and they were the only rats used in this experiment whose data failed to confirm to those obtained in the experiments with immature dogs described in part I. Assuming the correctness of the thesis suggested by the dog experiments and its applicability to the rat, we might expect that in these two rats lactose would not have a favorable influence on calcium utilization. Added confirmation of this idea was obtained by reexamination of the absorption data obtained in the preliminary series of rat experiments, namely, those in which a low-calcium diet and Bergeim's iron method were used. The weights of the four rats in this series, which had given data not in harmony with those yielded by the other animals, were found to range from 170 to 210 gm. On the other hand, the remaining six rats of the first series weighed from 90 to 140 gm., and gave data indicating a favorable effect of lactose on calcium utilization.

Although we realize that other factors may operate to cause the observed variations in our results, we believe our data indicate that a certain degree of immaturity of the animal must play a role. If we are correct in this belief, then it appears that extended comparative studies on the same rat, where it is intended that the animal serve as its own control, will not yield the same results at all times. This factor, which for the present we shall call 'degree of immaturity,' without attempting to define it more explicitly, may assist in explaining the contradictory results reported in the literature.

SUMMARY AND CONCLUSIONS

The possible influence of the presence of lactose in the diet on the utilization of calcium and phosphorus was studied by performing balance experiments in two young dogs and one mature animal. The ratios and the amounts of these two elements were carefully controlled. Likewise the effect of the

phospho-protein casein was compared with that of egg albumen.

The data obtained from the young animals indicated very definitely that the presence of lactose in the diet favorably influences the utilization of calcium and phosphorus; no such result was obtained with the mature dog, however.

Attempts were made by experiments on rats, using Bergeim's technique, to determine whether this favorable action of lactose is due to increased absorption from or diminished excretion into the intestine. It appears that the use of a low-calcium diet in tracing the absorption of this element is of practically no value because of the quantities of lime thrown into the alimentary tract by the digestive secretions. The data obtained from rats showed too great variation to be judged as entirely satisfactory. Nevertheless, they very definitely confirmed the dog experiments indicating that 'degree of immaturity' plays a role in determining the effect of lactose on the utilization of calcium by a given organism. These experiments on rats support, but do not prove, the view that lactose acts to diminish the excretion of calcium into the intestine.

LITERATURE CITED

AUSTIN, W. C., AND S. A. MATHEWS 1927 I. The effect of the parathyroid hormone on gastric secretion. II. The calcium content of gastric juice. Am. J. Physiol., vol. 81, p. 552.

BATEMAN, W. G. 1916 The digestibility and utilization of egg proteins. J. Biol. Chem., vol. 26, p. 263.

BAUER, W., F. ALBRIGHT AND J. C. AUB 1929 Studies of calcium and phosphorus metabolism; calcium excretion of normal individuals on low calcium diet; also data on a case of pregnancy. J. Clin. Invest., vol. 7, p. 75.

BERGHEIM, O. 1926 Intestinal chemistry. VII. The absorption of calcium and phosphorus in the small and large intestines. J. Biol. Chem., vol. 70, p. 51.

BRIGGS, A. P. 1924 Some applications of the colorimetric phosphate method. J. Biol. Chem., vol. 59, p. 255.

CANNON, P. R., AND B. W. MCNEASE 1923 Factors controlling intestinal bacteria; the influence of hydrogen ion concentration on bacterial types. J. Infect. Dis., vol. 32, p. 175.

CLARK, E. P., AND J. B. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

COWGILL, G. R. 1923 An improved procedure for metabolism experiments. J. Biol. Chem., vol. 56, p. 725.

CRUICKSHANK, R. 1928 The experimental transformation of the intestinal flora. Brit. J. Exp. Path., vol. 9, p. 318.

DRAGSTEDT, L. R., AND S. C. PEACOCK 1923 Studies on the pathogenesis of tetany. I. The control and cure of parathyroid tetany by diet. Am. J. Physiol., vol. 64, p. 424.

GROSS, E. G. 1927 The effect of lactose on the calcium-phosphorus balance of dogs. Am. J. Physiol., vol. 80, p. 661.

INOUYE, T. 1924 Experimental tetany and diet. Am. J. Physiol., vol. 70, p. 524.

KLINE, O. L., J. A. KEENAN, C. A. ELVEHJEM AND E. B. HART 1932 Lactose in nutrition. J. Biol. Chem., vol. 98, p. 121.

MARRIOTT, W. M., AND C. G. L. WOLF 1905–1906 The determination of small quantities of iron. J. Biol. Chem., vol. 1, p. 451.

MCCRUDDEN, F. H. 1910 The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine and feces. J. Biol. Chem., vol. 7, p. 83.

———— 1911 The determination of calcium in the presence of magnesium and phosphates. The determination of calcium in the urine. J. Biol. Chem., vol. 10, p. 187.

MITCHELL, H. S., AND W. M. DODGE 1935 Cataracts in rats fed on high lactose rations. J. Nutrition, vol. 9, p. 37.

NEUMANN, A. 1902 Alkalimetrische Bestimmung der Phosphorsäure unter Benutzung der Säuregemisch-Veraschung. Zeit. f. physiol. Chem., vol. 37, p. 129.

OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., vol. 37, p. 557.

RETTGER, L. F., AND H. A. CHEPLIN 1923 A treatise on the transformation of the intestinal flora with special reference to the implantation of Bacillus acidophilus. New Haven.

ROBINSON, C. S., AND C. W. DUNCAN 1931 The effect of lactose and the acid-base value of the diet on the hydrogen ion concentration of the intestinal contents of the rat and their possible influence on calcium absorption. J. Biol. Chem., vol. 92, p. 435.

ROBINSON, C. S., C. F. HUFFMAN AND M. F. MASON 1929 The results of the ingestion of certain calcium salts and of lactose. J. Biol. Chem., vol. 84, p. 257.

SMITH, A. H., AND F. C. BING 1928 Improved rate of growth of stock albino rats. J. Nutrition, vol. 1, p. 179.

WALCH, E. L., AND A. C. IVY 1927–1928 Calcium excretion by the alimentary tract. Proc. Soc. Exp. Biol. and Med., vol. 25, p. 839.

THE TOXICITY OF HIGH-GLIADIN DIETS. STUDIES ON THE DOG AND ON THE RAT [1]

DANIEL MELNICK [2] AND GEORGE R. COWGILL
Laboratory of Physiological Chemistry, Yale University, New Haven

(Received for publication April 3, 1937)

In the course of an investigation of the influence of diet upon the regeneration of serum protein in the dog (Melnick, Cowgill and Burack, '36), it was essential to determine previously for each of the tested proteins the minimal quantity required to secure nitrogen equilibrium. The source of the proteins and vitamin adjuvants, the construction of the diets and the nitrogen balance studies, conducted with lactalbumin, serum protein, casein or gliadin as the sole protein in the rations, are described elsewhere (Melnick and Cowgill, '37). When gliadin is fed as the sole source of dietary protein, there is a deficiency with respect to the essential amino acid, lysine. Probably it is for this reason that we found it necessary to feed the protein at a very high level of intake before nitrogen equilibrium was attained. Indeed, fully three times as much gliadin as compared with lactalbumin was essential for satisfying the general nitrogen requirements of the organism (Melnick and Cowgill, '37). In the course of these experiments certain unexpected toxic reactions were noted in the dogs subsisting on the high-gliadin diets. Obviously, the phenomena were not due to the amino acid deficiency characteristic of the protein, for it was with the high-gliadin diets alone that the reactions were observed. Only when the protein was consumed in amounts approaching and surpassing the minimal

[1] The expenses of this investigation were defrayed by a grant from the Research Fund, Yale University School of Medicine and by an Alexander Brown Coxe Fellowship Grant.

[2] Alexander Brown Coxe Fellow, 1936–1937.

value for nitrogen equilibrium were these manifest. Is it possible that the ingestion of a foodstuff, either in the form of protein or its constituent amino acids, at a level of intake satisfactory for the general nitrogen requirements of the organism can be toxic? The important implications of such a possibility warrant careful study.

The phenomena. On the seventh to the ninth day of the dietary regime the dogs fed a high-gliadin diet exhibited a peculiar behavior. The initial reaction may be characterized by the term 'running fits.' The dog suddenly begins to act in a strange manner and apparently is greatly frightened. He looks around and begins barking as if in severe pain. The animal then runs wildly, hurling itself blindly against the walls of the cage. This occurs generally for from 2 to 10 minutes. Subsequently, during the recovery period, the animal appears bewildered but otherwise normal. In some cases one attack may be followed immediately by another.

The more intense reaction which comes on with prolonged feeding of the high-gliadin diet bears a striking resemblance to epileptic convulsions. The animal first goes through the initial stage described above. This is followed by intense foaming at the mouth, the animal running around wildly attempting to 'catch its breath.' Suddenly the head is pulled to one side and, like the rest of the body, is held stiffly. The animal drops heavily on its side with all the muscles of the body undergoing rapid, fixed, rhythmic contractions. The dog then hurls itself furiously about the cage. With the return of normal respiration the animal lies relaxed and appears to be in a stupor. Several minutes later the animal is on its feet, apparently normal. Usually the frothy saliva is colored with blood due to the tongue or cheeks having been bitten. The bladder and the bowels are frequently emptied during these convulsions. We have noted several such seizures in the same animal following each other in rapid succession. At first, the toxic reactions were observed just after the gliadin diets were offered to the animals or during the time the rations were being ingested. Subsequently, convulsions were noted to occur

irrespective of the feeding schedule and, therefore, were interpreted as being neither conditioned nor due to the presence of a recently ingested meal in the stomach. The fact that these attacks are related in some way to the protein used in the diet is obvious from table 1.

The phenomena are not due to intestinal parasites. Dog no. 9 never showed these reactions, since the gliadin was only fed at that level constituting 13% of the caloric intake. Dog no. 10 was fed the gliadin diet at 7.45, 10.6 and 22% levels consecutively, developing convulsions only with the last-named

TABLE 1

Toxic effects of feeding high-gliadin diets

DOG NO.	BREED (SEX)	WEIGHT[1]	PER CENT OF CALORIES IN THE FORM OF GLIADIN					
			7.45	10.6	13.0	16.0	22.0	25.0
		kg.						
9	Spitz (female)	8.00	----	----	N.R.	----	----	----
10	Fox-terrier (female)	8.95	N.R.	N.R.	----	----	R.F. and E.C.	----
11	Bulldog (female)	7.76	----	N.R.	----	R.F. and E.C.	----	R.F. and E.C.
13	Mongrel (female)	9.23	----	----	N.R.	----	----	R.F.

[1] These values are calculated for the animals when adjusted to an optimal nutritive condition, as estimated by the nutritive index formula (Cowgill, '28).

----, indicates gliadin not fed at that caloric level.
N.R., indicates no reaction.
R.F., indicates 'running fits.'
E.C., indicates epileptiform convulsions.

ration. Dog no. 11 was fed the 10.6 and 16.0% diets consecutively with convulsions occurring only with the latter diet. Inasmuch as worms, especially tape- and hook-worms (Hutyra and Marek, '26; Hall, '25) have been reported to cause convulsions in dogs, attempts were made to determine whether intestinal parasites constituted the etiologic agent. Dogs nos. 10, 11 and 13 were given vermifuge, arecoline [3] being employed for removing the tape-worms and tetrachlorethylene [3] for the

[3] Obtained from the Parke, Davis and Co., Detroit, Mich.

round-worms. Dog no. 10 was then fed a stock diet consisting of a mixture of natural foods and never did develop any untoward reactions. Dogs nos. 11 and 13 were fed gliadin at the 25.0% level and were observed to show the characteristic symptoms after the eighth day on this diet. The feces of these dogs were free of tape-worm eggs and contained a small number of hook-worm ova.[4] Experimentation with these animals was discontinued. It was surprising to note that fully a week was required before these dogs, subsisting on the stock diet, ceased showing an occasional convulsion.

These seizures are not due merely to a high-protein diet per se. This is obvious, when it is considered that the casein diet (Cowgill, '28), commonly used in this laboratory, which contains protein to the extent of approximately 28% of the caloric intake, has never caused similar reactions in dogs, including our own experimental animals even when subsisting on the diet for as long as 18 months.

The toxic reactions are not due to a significant uremia. The behavior of the animals subjected to the high-gliadin diets suggested that there might be an accumulation of some toxic substance, and that the disappearance of the reaction occurred only when this substance was reduced below a threshold value. Gliadin is composed of as much as 43.7% glutamic acid (Mendel, '23). This amino acid, when introduced parenterally, has been shown to be toxic for rabbits (Johnston and Lewis, '28). In the case of man, Lewis and co-workers ('18) have reported that glutamic acid ingestion is productive of nausea, general malaise and oliguria. Our dogs all showed an associated oliguria, the urine excretions falling to from one-third to one-half of their normal volumes. This finding is all the more significant in view of the fact that the dogs subsisted on a high-protein diet and such diets, by giving rise to nitrogenous end-products, tend to exert a diuretic effect. MacKay ('33) has noted that the administration of glutamic acid to

[4] The writers acknowledge their indebtedness to Dr. Telford W. Workman for his assistance in investigating the possibility of intestinal parasites being responsible for the appearance of the seizures noted.

rats which have been maintained upon a basal diet containing casein, results in renal hypertrophy; the maximum effect was observed in the relatively short period of 10 days. Indeed, certain amino acids have been reported (Kotake, '34) to cause nephrosis, when injected intraperitoneally. Glutamic acid and cystine appeared to compensate each other since injections of both resulted in no nephrosis. Contrary to these suggestive findings, the relative non-toxicity of glutamic acid as contrasted with that of tyrosine and cystine has been reported (Sullivan, Hess and Sebrell, '32; Lillie, '32). Thus, the toxicity of the gliadin, observed in the present study, may perhaps be due to glutamic acid. However, in view of our own rat experiments and the additional tests on the dog, cited below, this does not seem to be an adequate explanation.

Seeking confirmatory evidence, three additional dogs were fed the 22% gliadin diet at the usual level of intake of 70 calories per kilogram of body weight together with the vitamin adjuvants. Determinations were made of the blood non-protein nitrogen (Folin and Wu, '19) before and after the use of this diet. The results of this study indicated that the convulsions could not be attributed to a significant uremia. The non-protein nitrogen values before and after the administration of the gliadin ration were essentially normal. In all cases the reactions subsequently disappeared when the animals were fed stock diets with no vermifuge treatments.

Experiments with the rat. Numerous investigators have fed rats artificial 'synthetic' diets in which gliadin constituted practically the sole source of protein. No convulsive reactions were ever reported. This may be due to a species difference or due to the fact that the ingested gliadin constituted only 11 to 13% of the caloric intake. In the present study attempts have been made to determine whether high-gliadin diets are toxic for rats.

The possibility that the substance, active in producing the seizures noted in our dogs, may be merely a contaminant of the gliadin preparation was also studied. The investigations of Franke ('34) of a toxicant occurring naturally in certain

samples of plant foods have indicated that this substance is carried by the protein fraction. Subsequent studies (Franke and Painter, '36) with the gluten protein from toxic wheat have shown that selenium in organic combination with the protein is responsible for the toxicity of the foodstuff. The gliadin employed in our investigations was prepared from gluten flour [5] (Nolan and Vickery, '36). In view of these facts, might it not be possible that selenium may be present as a contaminant of the gliadin and thus be the toxic substance in the protein preparation? However, analyses [6] of the gliadin indicated that our preparation was free from selenium. With respect to other possible inorganic contaminants of the protein, it is pertinent to point out that the preparation contained only 0.15% ash. Furthermore, the gliadin was isolated by means of a 75% alcoholic extraction of the gluten flour, a procedure which tends to leave the inorganic salts behind with the insoluble residue.

The possibility of some other toxic substance adhering to the gliadin preparation was investigated in the rat, using growth data as the criteria. Thirty-three male rats, weighing within 40 to 50 gm. at weaning, were employed. These animals were weaned at 21 days of age and distributed into six groups. In no case were two rats from the same litter placed in the same group. The animals were fed artificial 'synthetic' diets containing casein,[7] gliadin, or gliadin plus lysine as the sole source of protein. The proteins were fed at two levels, '18%' and '36%' (by weight) of the dietary mixture. The casein contained 13.37% nitrogen; the gliadin, 14.81%. These analyses indicate that both proteins were 84.1% pure (Jones, '31). Thus, the percentages of pure protein in the dietary mixture were actually 15.14 and 30.28%, and constituted 12.4 and 25.6% of the caloric intake, respectively. For the sake of

[5] Obtained from the Battle Creek Food Co., Battle Creek, Mich. It is reported as 'containing 85% gluten.'

[6] We are indebted to Dr. Alvin L. Moxon for these analyses. These determinations were carried out in Franke's laboratory, South Dakota State College of Agriculture and Mechanic Arts, Brookings, S. D.

[7] Obtained from the Lister Bros., New York, N. Y.

convenience, the diets will be referred to as the 18 and 36% protein rations. One of the 18% gliadin diets was supplemented with *dl*-lysine dihydrochloride [8] to the extent of 1.62% of the dietary mixture. Osborne and Mendel ('14) have shown that 'normal' growth in the rat may be promoted when a maintenance ration containing gliadin as the sole protein is supplemented with lysine. In their work gliadin was fed at an 18% level by weight and lysine added equivalent to 3% of the gliadin, i.e., 0.54% of the total ration. Since only one-third of *dl*-lysine dihydrochloride (Berg, '36) is physiologically active with respect to promoting growth, we have supplemented the gliadin diets with three times that quantity, namely, 1.62% of the ration.

In order to compare favorably the experiments with the rat with those conducted on the dog, the diets in both studies were constructed on the kilo-unit (Cowgill, '23; Melnick and Cowgill, '37). The vitamin adjuvants were the same but the quantities given to the rat were different from those fed the dog. The daily administration to the rat of 200 mg. of a rice polishing extract [9] and 200 mg. of liver extract no. 343 [10] satisfied the vitamin B (B_1) and G (B_2) requirements, respectively. For vitamins A and D, 20 mg. of a cod liver oil concentrate [11] were administered every 4 days. These quantities of the vitamin adjuvants have been found to supply abundantly the needs of the growing and adult rat.[12]

From the time of weaning all the rats were fed the 18% casein diet plus the vitamin supplements in order to allow the animals adequate time for adjustment to a new dietary regime. After each rat had consumed 250 calories of this ration, it was fed 650 calories of one of the six diets employed in this study. The animals were weighed daily. At the end of the experimental period the rats were killed. The kidneys were

[8] Obtained from the Eastman Kodak Co., Rochester, N. Y.

[9] 'Ryzamin,' obtained from the Burroughs Wellcome Co., Tuckahoe, N. Y.

[10] We are indebted to the Eli Lilly Co., Indianapolis, Ind., for a generous supply of this material in powdered form.

[11] Kindly furnished by the Health Products Corp., Newark, N. J.

[12] Personal communication from Aline U. Orten.

removed and weighed; the average weights were correlated with surface area, estimated from the average body weight values (Carman and Mitchell, '26). Table 2 summarizes the experimental findings.

A consideration of the data presented in the table indicates that the protein, isolated from wheat gluten and used in the present study, was a good gliadin preparation as judged by

TABLE 2

Summary of the data obtained with rats subsisting on artificial 'synthetic' diets containing casein, gliadin or gliadin plus lysine as the sole source of protein

GROUP (NUMBER OF RATS)	DIET	AVERAGE FOOD INTAKE PER DAY	AVERAGE BODY WEIGHT			KIDNEY WEIGHTS [1]		
			Initial	End of period I	End of period II	Range	Average	Per 100 sq.cm. of surface area
		gm.	gm.	gm.	gm.	gm.	gm.	gm.
I	18% casein	6.3	44	71				
(8)	18% casein	8.6			126	0.411–0.549	0.464	0.162
II	18% casein	6.1	44	71				
(4)	18% gliadin	5.4			70	0.306–0.343	0.331	0.171
III	18% casein	6.1	45	73				
(4)	18% gliadin + 1.62% *dl*-lysine · 2 HCl	7.1			101	0.382–0.413	0.401	0.162
IV	18% casein	6.3	46	74				
(7)	36% casein	9.0			156	0.566–0.743	0.632	0.192
V	18% casein	6.3	45	75				
(5)	36% gliadin	5.7			79	0.379–0.460	0.422	0.201
VI	18% casein	6.3	46	75				
(5)	36% gliadin + 1.62% *dl*-lysine · 2 HCl	7.8			129	0.513–0.559	0.542	0.186

[1] The average weight of the two kidneys of each rat were used in calculating these values.

the available criteria. It was not contaminated to any appreciable extent by any other proteins found in wheat gluten. This is evident when comparisons are made of the rate of growth of rats subsisting on the 18% gliadin diet with that noted with the control animals fed the 18% casein diet. The ingestion of the gliadin ration supplemented with lysine promoted growth at the rate of 1.5 gm. per day. No obvious toxic

symptoms were noted in these rats. These were not expected since the gliadin constituted only 12.4% of the caloric intake.

Rats fed a 36% gliadin diet were stunted almost to the same extent as those subsisting on the 18% ration. This finding is significant because dogs subsisting on a 36% gliadin diet (25.6% of the caloric intake) will attain nitrogen equilibrium (Melnick and Cowgill, '37). Gliadin does contain 0.63% of lysine (Mendel, '23), but this is not sufficient to permit growth in rats fed the usual 18% protein diet. However, with respect to the attainment of nitrogen equilibrium in adult animals, it appears that sufficient lysine was ingested, when the dogs consumed the high-gliadin diet, associated with the positive balances recorded (Melnick and Cowgill, '37). The possibility existed, therefore, that rats fed a 36% gliadin diet might exhibit appreciable growth. The experimental findings proved that such is not the case. Could it be possible that the inhibition of growth was due not to the lysine deficiency but to the toxic principle associated with the gliadin preparation? Supplementation of the gliadin ration with lysine resulted in a normal rate of growth, 3.1 gm. per day. The amount of lysine added to the 36% gliadin ration was identical with that supplementing the 18% diet, but the growth response in rats ingesting the former diet was actually two times greater. Apparently the lysine content of gliadin, although insufficient in itself to permit growth in rats fed the 36% protein diet becomes significant when combined with the lysine supplement: it is nutritionally significant. This finding appears to confirm our suggestion that the attainment of nitrogen equilibrium by the dog subsisting on the high-gliadin diet is due to the ingestion of sufficient lysine as part of the protein intake. No obvious 'toxic' symptoms were observed with the rats subsisting on the high-gliadin diet other than inhibition of growth, and this was corrected by adding lysine to the diet.

The possibility that sufficient glutamic acid may be ingested as part of the protein intake to cause a nephrosis in rats subsisting on our gliadin diets was also studied. The kidney weights (table 2) indicate no significant atrophy or hypertrophy of that organ. This is especially true if these values are

correlated with the surface areas of the animals. The slightly larger kidney weights recorded for the animals subsisting on the 36% protein rations are in accordance with the finding that the ingestion of high-protein diets by the rat results in renal hypertrophy (Osborne, Mendel, Park and Winternitz, '27). Histological study [13] indicated that the kidneys of rats fed the 36% gliadin diet were normal. Furthermore, the urines obtained from these animals were free from protein.

The toxicity of the high-gliadin diets may be due to a protein sensitization.[14] Subsequent to the protein minima studies conducted with dogs nos. 10, 11 and 13, the animals were removed from the metabolism cages and returned to the dog house. Approximately 15 months later, dog no. 10 was brought back to the laboratory to be used as the experimental subject in another investigation. As soon as the animal entered the laboratory, a typical seizure occurred, similar in character to those noted previously. While this dog had been kept in the dog house, no convulsive reactions had ever been noted. Inasmuch as gliadin was being prepared in the adjoining laboratory, the seizure was suspected of being allergic in character. In this connection it is pertinent to point out that the initial toxic reactions, observed with the dogs subsisting on high-gliadin diets, occurred at the time when the animals were being fed.

Evidence that idiopathic epilepsy may be a sensitization disease has been advanced by Howell ('23) and Miller ('24). Since the symptoms observed in our animals were not typical of anaphylactic shock in the dog (Melnick, Burack and Cowgill, '36), we had neglected previously to investigate the possibility that the epileptiform reactions may be due to the dogs having been sensitized to gliadin. Subsequent investigations were conducted in order to test the validity of such a hypothesis.

[13] We are indebted to Dr. Harry M. Zimmerman of the Department of Pathology, Yale University School of Medicine, for interpretations of the kidney sections.

[14] We acknowledge our indebtedness to Dr. Louis Weinstein of the Department of Bacteriology, Yale University School of Medicine, for his generous assistance during this phase of the investigation.

Preliminary tests, passive transfer skin tests on a non-sensitized dog and the intravenous injections of a gliadin solution into guinea pigs which had been passively sensitized with the serum from dog no. 10, suggested that the toxicity of the high-gliadin diets may be due to a protein sensitization. The protein was employed as a 4.4% solution in 75% alcohol.[15] It is not considered advisable to inject intravenously such a solution since, in addition to the toxicity of the strong alcoholic solution, the gliadin is immediately precipitated on coming into contact with the blood. However, if an equal volume of a $\frac{N}{10}$ sodium hydroxide solution is first added to the gliadin solution, the further addition of 8 parts of a 0.5% sodium chloride solution does not precipitate the protein. In the subsequent tests all the gliadin solutions used for intravenous injections were prepared according to this procedure. The apparatus and technique used for the injection of the solution were the same as that employed for the reinjection of the red blood cell suspensions into our dogs subjected to plasmapheresis (Melnick and Cowgill, '36). This point is mentioned since toxic reactions are frequently associated with intravenous injections due simply to faulty technic. We do not believe such an explanation can be offered for our observations.

A passive transfer test was conducted using a normal non-sensitized dog as the test animal. Dog no. 10 was bled 160 cc.; to this blood a 3% sodium citrate solution was added until the final concentration of the anticoagulant was 0.3%. Approximately 90 cc. of the citrated plasma (75 cc. of whole undiluted plasma) were injected intravenously into dog no. 53, a male terrier, weighing 7.0 kg. Twenty-four hours later the diluted gliadin solution, prepared according to the directions given above, was injected into the jugular vein at the rate of approximately 50 cc. per minute. Respiration and the heart rate were slightly stimulated. After 80 cc. of the solution were injected, the animal was given a rest period of 3 minutes; this was then followed by the injection of an additional 80 cc. During the second injection, there was a marked fall in the blood

[15] Analyses of the protein indicated a purity of 84.1%, so that the true concentration of gliadin in the alcoholic solution was actually 3.7%.

pressure, labored respiration and a pronounced cardiac arrhythmia. Almost simultaneously with the conclusion of the injection, respiration and the heart beat stopped. The injection of 0.25 cc. of an adrenalin solution [16] into the heart resulted within 1 to 2 minutes in the complete recovery of the animal.

The above experiment was repeated on another dog, but this time the animal, dog no. 54, a male terrier also weighing 7.0 kg., was not previously sensitized by plasma injections from dog no. 10. The response obtained with this animal resembled that recorded for dog no. 53. However, respiration and the heart beat did not cease; the animal recovered quickly without therapy of any kind.

It seemed to us that the differences in the degree of shock noted in the above experiments could be differentiated simply on the basis of dose. To test such a hypothesis, dogs nos. 10, 11 and 13, presumably actively sensitized to gliadin, were given an intravenous injection of the diluted protein solution. The doses, which were also given in two injections, were calculated on the basis of body weight. Thus, dog no. 10 received approximately 210 cc.; dog no. 11, 180 cc.; and dog no. 13, 210 cc. Precisely the same response, as that noted in the experiment conducted with dog no. 53, was obtained with dog no. 10. The intracardiac injection of adrenalin caused complete recovery from the shock reaction. In the case of dog no. 11, it was deemed advisable to allow more than 3 minutes between injections so as to compensate for any possible latency in the appearance of shock. Within 5 to 6 minutes after the first injection of 90 cc. of solution, the dog vomited and defecated; this was followed by complete prostration similar in character to that recorded with dog no. 54. The animal finally recovered without therapy and appeared quite normal 15 minutes after the injection. The injection of the remaining part of the dose resulted in no obvious harmful effects other than slight intoxication. Dog no. 13 failed to show any response to

[16] Adrenalin chloride (1: 1000), obtained from the Parke, Davis and Co., Detroit, Mich.

the injections other than symptoms of mild intoxication, such as a mild ataxia of about 10 minutes' duration.

If we accept the hypothesis that the toxicity of high-gliadin diets is due to a protein sensitization, the results obtained with dog no. 11 are easily explained. Apparently the initial injection of the protein solution more or less completely saturated the antibodies with the antigen. Thus the second injection was followed by no adverse effects, although the amount of foreign protein injected into the animal was doubled. As was pointed out in table 1, dog no. 13, in contrast to the other animals, exhibited only the mild, toxic reaction when fed the high-gliadin diet. Thus, the negative response to the intravenous injection of the gliadin solution in the case of this animal may not be inconsistent with the results recorded with dogs nos. 10 and 11. However, when the results obtained with this animal are compared with the observations recorded with dog no. 54, which had never subsisted on the high-gliadin diets, it appears that dosage may play some part in determining the character of the response to the intravenous injections of the protein solution here described. This conclusion is also supported by the fact that dog no. 10, presumably actively sensitized to gliadin, required a dose comparable to that injected into dog no. 53 before shock was manifest.

Although dog no. 54 showed symptoms of shock similar in character to those recorded with dogs nos. 53 and 10, the animal quickly recovered without the aid of adrenalin therapy. The possibility existed that, if a greater volume of the gliadin solution had been injected, the shock reaction would have been fatal. This was confirmed by the injection of a relatively large amount of the protein solution into another normal dog. This animal, dog no. 55, a female mongrel with an optimal weight of 12.6 kg., received 350 cc. of the gliadin solution intravenously. This dose in terms of body weight was approximately 20% greater than those administered to the other dogs. The first part of the injection was tolerated satisfactorily. After the usual 3-minute rest period the remainder of the dose was injected. The animal was thrown into profound shock.

In order to be sure that there would be no spontaneous recovery, we waited approximately 1 to 2 minutes before resorting to the use of adrenalin. Three 0.25 cc. injections failed to save the animal.

It would seem that the correlation of effect with dose should rule out anaphylaxis as the mechanism responsible for the shock reactions. However, it should be remembered that the solutions as injected contained only a small amount of protein, namely, 3.7 mg. per cubic centimeter. The possibility that some other factors might be responsible for the shock reactions was investigated. According to the studies conducted by Hyatt ('19), the alcohol could not be the cause of the appearance of shock. This was confirmed by injecting into each of the five dogs (nos. 53, 54, 10, 11 and 13) the alcoholic, alkaline, saline solution, prepared in the same manner as described but this time with no gliadin added. The volumes injected into each dog were actually 20% greater than those employed when gliadin was administered. These experiments were repeated a week later but this time casein [17] was injected instead of gliadin. It was concluded from these tests that the symptoms of profound shock noted in our dogs were due to the intravenous injection of the protein, gliadin, and not due to the character of the solvent or merely to the introduction of any foreign protein into the circulation.

In connection with these experiments, it is of interest to note that Seibert and Mendel ('23) found that most casein preparations, when injected intravenously into rabbits, cause a marked rise in body temperature, whereas gliadin is apparently without effect. However, the amounts of protein so administered were exceedingly small; for the most part only 5 mg. of protein were injected into each animal. Nevertheless, the effects obtained in the present study as a result of the gliadin injections cannot be explained on the basis of dose alone. It is true

[17] A 0.44% solution of a casein preparation obtained from the Arlington Chemical Co., Yonkers, N. Y., was used. This particular sample contained 13.72% nitrogen which is indicative of an 86.2% purity (Jones, '31). Thus the true concentration of the protein in the alcoholic, alkaline, saline solution was actually 3.8 mg. per cubic centimeter.

that the adjustment of the pH of the gliadin solution to that of the blood results in a precipitation of the gliadin in the form of extremely fine particles which settle out very slowly. Thus, it seems possible that such occurs when the protein solution comes in contact with the blood. It also appears likely that under such conditions the formation of multiple emboli may occur. However, it is impossible to attribute the observed toxic reactions to such a cause. Dog no. 11 showed a typical shock reaction after the injection of only one-half of the calculated dose of the gliadin solution. If this effect is to be explained on the basis of the formation of multiple emboli, one should expect a second reaction when the remainder of the dose was injected. However, the administration of the second half of the protein solution, after the animal had recovered from the initial shock reaction, resulted in no obvious untoward effects other than a mild intoxication. Furthermore, the rapid and complete recoveries of dogs nos. 53 and 10 from shock, following intracardiac administration of adrenalin, also preclude the possibility that the toxicity of the protein solutions is due to the tendency for the formation of multiple emboli.

It seems to us that the evidence presented suggests that the toxicity of high-gliadin diets may be due to a sensitization of the animal to the protein. It is not unlikely that all dogs are more or less sensitive to gliadin. It may be that the feeding of rations containing excessive amounts of this protein to the dogs finally results in the absorption of certain fragments of the gliadin molecule, which in sufficient concentrations are capable of precipitating the epileptiform reactions described in the early part of this paper.

In the case of the rat, the feeding of the high-gliadin diet produced no 'toxic' effects other than an impairment of growth and this was corrected by adding lysine to the ration. If the seizures observed with the dogs can be attributed to a protein sensitization, then the negative effects obtained with the rat are consistent with the fact that this species is particularly non-sensitive to the injection of foreign protein. This belief

was confirmed by tests conducted on nine mature rats. These animals were injected by way of the femoral vein with varying doses of the gliadin solution, calculated on the weight basis to be in some cases actually twice as large as that used in the study with the dog. On the basis of these tests, it was concluded that the rat is undoubtedly more resistant than the dog to the intravenous administration of gliadin, and that any toxicity evidenced by the rat is probably due simply to the character of the solvent used.

Many investigations, in which gliadin was used as the sole source of protein in the ration, have been reported in the literature without any mention of a toxicity having been observed. This may be due to the fact that in all such experiments, with which we are familiar, the rat was used as the experimental animal. This species has been shown in the present study to be particularly resistant to the toxicity of high-gliadin diets. In the case of the dog both the cost of feeding the expensive gliadin rations to such a large animal and the fact that most dogs exhibit difficulty in consuming such diets have led investigators to refrain from using this species in feeding experiments involving the use of gliadin. During the ingestion of such rations this protein tends to 'gum up' and stick to the teeth. However, as we discovered (Melnick and Cowgill, '37), if the diet is first mixed with an equal part of water to form a loose pasty mass, no difficulties will be encountered in getting the dog to ingest its daily aliquot of the ration.

SUMMARY

In the present study it was possible to produce convulsive reactions in all of six dogs fed high-gliadin diets in which gliadin was the sole protein and furnished 16% or more of the caloric intake. Intestinal parasites were ruled out as possible cause of the toxic reactions. The fact that there was an initial lag in the appearance of gliadin toxicity and a delay in the disappearance of the seizures suggests the accumulation of some toxic substance during the gliadin feedings and its subsequent elimination. The increases in blood non-protein nitrogen were not sufficiently large to indicate that the con-

vulsions were uremic in nature. Various experiments tentatively suggest that this toxicity of the high-gliadin diets may be due to a protein sensitization.

The gliadin, isolated from a 75% alcoholic extract of gluten flour, contained only 0.15% ash. Selenium was not present as a contaminant of the protein.

Insofar as growth data are concerned, feeding experiments showed that the gliadin was free of any substance toxic for the growing rat. No convulsive reactions were observed in the rats subsisting on a gliadin diet in which the protein furnished as much as 25.6% of the caloric intake. Apparently susceptibility to gliadin toxicity is a species characteristic.

The rats, fed the 36% (by weight) gliadin diet, subsisted on a ration containing actually 13.2% glutamic acid. The ingestion of a dietary mixture, containing such a high concentration of this amino acid, appeared to be non-toxic for this species.

The nutritional significance of the lysine present in gliadin was emphasized. Rats fed an 18% or a 36% gliadin diet were stunted practically to the same extent. However, when both rations were supplemented with the same amount of lysine, growth was twice as rapid in those animals fed the 36% diet.

LITERATURE CITED

BERG, C. P. 1936 The availability of *d* (—) lysine for growth. J. Nutrition, vol. 12, pp. 671–676.

CARMAN, G. G., AND H. H. MITCHELL 1926 Estimation of the surface area of the white rat. Am. J. Physiol., vol. 76, pp. 380–384.

COWGILL, G. R. 1923 An improved procedure for metabolism experiments. J. Biol. Chem., vol. 56, pp. 725–737.

———— 1928 The energy factor in relation to food intake. Experiments on the dog. Am. J. Physiol., vol. 85, pp. 45–64.

FOLIN, O., AND H. WU 1919 A system of blood analysis. J. Biol. Chem., vol. 38, pp. 81–110.

FRANKE, K. W. 1934 A new toxicant occurring naturally in certain samples of plant foodstuffs. II. The occurrence of the toxicant in the protein fraction. J. Nutrition, vol. 8, pp. 609–613.

FRANKE, K. W., AND E. P. PAINTER 1936 Selenium in proteins from toxic foodstuffs. I. Remarks on the occurrence and nature of the selenium present in a number of foodstuffs or their derived products. Cereal Chem., vol. 13, pp. 67–70.

HALL, M. C. 1925 Parasites and parasitic diseases of dogs. U. S. Dept. Agr., circular no. 338, 29 pp.

HOWELL, L. P. 1923 Epilepsy and protein sensitization. Ohio State Med. J., vol. 19, pp. 660–662.

HUTYRA, F., AND J. MAREK 1926 Special pathology and therapeutics of the diseases of domestic animals. II. Diseases of the digestive organs. (Editors, J. R. Mohler and A. Eichhorn.) viii, 850 pp. Alexander Eger, Chicago, Ill.

HYATT, E. G. 1919 The action of alcohol on the heart and respiration. J. Lab. Clin. Med., vol. 5, pp. 56–58.

JOHNSTON, M. W., AND H. B. LEWIS 1928 Comparative studies of the metabolism of amino acids. I. Changes in the non-protein nitrogenous constituents of the blood following administration of amino acids. J. Biol. Chem., vol. 78, pp. 67–82.

JONES, D. B. 1931 Factors for converting percentages of nitrogen in food and feeds into percentages of proteins. U. S. Dept. Agr., circular no. 183, 21 pp.

KOTAKE, Y. 1934 The metabolism of proteins and amino acids. Ann. Rev. Biochem., vol. 3, pp. 193–208 (see p. 202).

LEWIS, H. B., M. S. DUNN AND E. A. DOISY 1918 Studies in uric acid metabolism. II. Proteins and amino acids as factors in the stimulation of endogenous uric acid metabolism. J. Biol. Chem., vol. 36, pp. 9–26.

LILLIE, R. D. 1932 Histopathologic changes produced in rats by the addition to the diet of various amino acids. U. S. Pub. Health Repts., vol. 47, pp. 83–93.

MACKAY, E. M. 1933 Factors which determine renal weight. XIV. The relative influence of amino, urea and protein nitrogen in the diet. J. Nutrition, vol. 6, pp. 157–161.

MELNICK, D., E. BURACK AND G. R. COWGILL 1936 Development of incompatibilities in dogs by repeated infusions of red blood cells. Proc. Soc. Exp. Biol. and Med., vol. 33, pp. 616–621.

MELNICK, D., AND G. R. COWGILL 1936 A quantitative technique for performing plasmapheresis. J. Exp. Med., vol. 64, pp. 865–876.

———— 1937 The protein minima for nitrogen equilibrium with different proteins. J. Nutrition, vol. 13, pp. 401–424.

MELNICK, D., G. R. COWGILL AND E. BURACK 1936 Influence of diet upon the regeneration of serum protein. II. The potency ratios of serum protein, lactalbumin and casein, and the effect of tissue protein catabolism on the formation of serum protein. J. Exp. Med., vol. 64, pp. 897–920.

MENDEL, L. B. 1923 Nutrition: The chemistry of life. xii, 150 pp. Yale University Press, New Haven, Conn.

MILLER, J. L. 1924 Evidence that idiopathic epilepsy is a sensitization disease. Am. J. Med. Sci., vol. 168, pp. 635–641.

NOLAN, L. S., AND H. B. VICKERY 1936 Preparation of gliadin and zein. Proc. Soc. Exp. Biol. and Med., vol. 35, pp. 449–451.

OSBORNE, T. B., AND L. B. MENDEL 1914 Amino-acids in nutrition and growth. J. Biol. Chem., vol. 17, pp. 325–349.

OSBORNE, T. B., L. B. MENDEL, E. A. PARK AND M. C. WINTERNITZ 1927 Physiological effects of diets unusually rich in protein or inorganic salts. J. Biol. Chem., vol. 71, pp. 317–350.

SEIBERT, F. B., AND L. B. MENDEL 1923 Protein fevers with special reference to casein. Am. J. Physiol., vol. 67, pp. 105–123.

SULLIVAN, M. X., W. C. HESS AND W. H. SEBRELL 1932 Studies on the biochemistry of sulfur. Preliminary studies on amino acid toxicity and amino acid balance. U. S. Pub. Health Repts., vol. 47, pp. 75–83.

THE EFFECTS OF DEFICIENCY OF PHOSPHORUS ON THE UTILIZATION OF FOOD ENERGY AND PROTEIN [1]

E. B. FORBES [2]

Institute of Animal Nutrition, Pennsylvania State College, State College

(Received for publication April 13, 1937)

INTRODUCTORY

A continuing program of studies is in progress, at the Institute of Animal Nutrition of Pennsylvania State College, in which is sought the reaction of growing albino rats to deficiencies of single nutritive principles; the experimental procedure, which is original to this series, being the same in each unit.

The characteristics of this procedure, which is called 'the body balance method,' are that the complete accounting for the disposal of the food energy is accomplished as follows: 1) The energy of the body gain is measured as the difference between the energy of the bodies of two groups of animals, one of which has received an experimental diet, and the other has been selected to represent the experimental group in its initial status; 2) the energy of the excreta of the experimental group is directly determined; and 3) the heat production is measured as the gross energy of the food minus the energy of the excreta and of the body gain.

The mathematical validity of this procedure is safeguarded especially by the employment of large numbers of

[1] Authorized for publication on April 9, 1937, as paper no. 767 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] This paper is based on experiments, conducted under the author's direction, by Orme J. Kahlenberg, R. W. Swift, Alex Black, Winfred W. Braman (deceased) and Max Kriss.

experimental subjects, fed for an extended period of observation; and when it is used as the basis of comparison of the energy values of diets, these are fed with the paired method of control.

Consideration of the nature and of the constancy of the data on which this procedure is based show that, as a method of measurement of the total, unfactored heat production, it is characterized by a high degree of validity.

An incident in the current trend of thought relating to the broader economic problem of nutrition, as resulting from new trends of the times, is that minimum nutritive requirements of man, and of domestic animals, have lost much of their former apparent importance; while optimum nutritive requirements have attained new practical significance.

In other words, a new economy of superabundance is superseding an age-old 'economy of minimum;' and in this relation it is pertinent to investigate the questions as to what are the limits of useful phosphorus intake, for the growing albino rat, and what are the evidences of phosphorus deficiency.

To specify the functions of phosphorus in animal metabolism is to enumerate the vital processes of the body; the integrity of these processes being maintained by safety provisions of various kinds, including stores of phosphorus, mainly in the skeleton, of extents commensurate with the demands likely to be made upon them, and also with the supreme importance of the animal economy as a whole.

By virtue of these safeguards, the vital processes of the organism are insured against disturbance from variations likely to occur in the phophorus intake; and, within limits, such variations are presumably of consequence to the animal only as causing fluctuations in the body reserves of phosphorus—without effects on the vital processes. These safeguards, however, may be overtaxed—with the result that metabolism is affected.

The effects of phosphorus deficiency, therefore, may be any such disturbance of metabolism as is precipitated—immediately or later—by insufficient intake of this element.

In view of the conception of complete nutrition, and the obvious facts that no essential nutrient can be more essential than another; but that there are inevitable differences in the relative extent of the body reserves of essential nutrients, and in the rapidity with which they may be physiologically mobilized; the noticeable effects of differences in the level of phosphorus supply may not be definitely specific, in the sense of being obviously related either to its chemical nature or to the more conspicuous of its functions, but may be general in character. In fact, in a critical sense, there is no such thing as phosphorus metabolism; there is only metabolism in general, in which phosphorus is involved—in a vast diversity and complication of ways.

In the conduct of this series of experiments, therefore, the observations made are related to each other only as results of studies of single nutritive deficiencies, all having been obtained by the same method.

The background of the present study may be considered to be the vast existing knowledge of the fundamental relations of phosphorus in the metabolism of the tissues and organs of the body; but this investigation itself deals only with considerations of phosphorus deficiency in the albino rat, as affecting the economy of utilization of food energy and protein for maintenance and body increase.

Numerous students of animal feeding have drawn conclusions of fundamental implication, as to the effects of phosphorus deficiency, from simple, incompletely controlled feeding experiments. In many cases the results seem so obvious as to render superfluous the more critical investigation of the subject; but attempts to determine the facts more certainly have shown that very few experiments, in the field specified, have been conducted with sufficiently critical control to demonstrate fundamental truths; and that there is need for further investigation by intensive methods—especially to answer questions as to the degree to which the phosphorus intake may be varied without disadvantage, and as to the ways in which, and the extents to which, phosphorus

deficiency may affect the utilization of food energy and protein. With these considerations the present study is concerned.

The scope of this investigation and the character of the findings, seem not to justify a general review of the literature of phosphorus metabolism, but a few of the later studies, which throw light on the subject as considered, will be mentioned.

Phosphorus deficiency occurs much more commonly with cattle than with other species; and, in this relation, is of economic importance, in the United States, in certain more or less definitely delimited regions characterized by low-phosphorus soils, in California, Florida, Kansas, New Mexico, Michigan, Minnesota, Wisconsin and Texas.

Theiler, Green and Du Toit ('24) concluded, from feeding experiments, that there was a lowered economy of feed utilization in aphosphorosis.

Eckles and Gullickson ('27) found that a plane of nutrition 1.2 times normal was required to maintain live weight in certain phosphorus deficient cows.

Woodman and Evans (30) observed that a deficiency of calcium and phosphorus in the diet of sheep did not depress the digestibility of the ration.

Eckles, Becker and Palmer ('26), and Eckles, Gullickson and Palmer ('32) concluded, from studies of phosphorus deficiency in cattle, by means of feeding experiments, that

> under extreme conditions, as in the area studied, a shortage of phosphorus becomes a limiting factor in the economical utilization of feeds, and in the growth of cattle. The data reported bear out the generally accepted fact that phosphorus is involved in the intermediary metabolism of carbohydrates.

The most inclusive studies that have been made of the effects of phosphorus deficiency on the performance of cattle are those of Riddell, Hughes and Fitch ('33, '34 a, b). Their observations, confirming much that had been learned by others, but also covering extensive new findings, may be summarized as follows: Phosphorus deficiency in dairy cows

causes failure and depravement of appetite, stiffness and muscular weakness, failure of nutrition as evidenced by loss in weight and excessive maintenance requirement, low inorganic phosphorus in the blood, failure of oestrum, low phosphorus but high calcium in the urine, negative phosphorus and calcium balances, rapid and shallow breathing, and increased pulse rate. There was no effect of phosphorus deficiency on body temperature, or on the digestibility or the metabolizability of the food; milk production was maintained at a relatively high level, in spite of phosphorus deficiency, and the animals on phosphorus deficient diet responded to dosage with monosodium phosphate by increase in appetite, in live weight, in lactation, and in improvement in the various pathological conditions resulting from phosphorus deficiency.

Riddell, Hughes and Fitch concluded, as had others previously, that, in consideration of the various conditions noted, phosphorus deficiency must cause increased heat production. They then measured the heat production of milking cows, as affected by phosphorus deficiency, by indirect calorimetry. The heat was computed from three 6-minute measurements of the oxygen consumption of the animal, 12 hours after feeding, by assuming a respiratory quotient of 0.82, the subject being in the lying position, which it had been taught to assume by 3 months' training.

The authors found a somewhat greater heat production with the phosphorus-deficient cows than with the controls. However, in consideration of the circumstances stated—especially that the total time of oxygen measurement was only one-eightieth of the day; and that these measurements were made 12 hours after feeding, and in only one position—which the animals took only as a result of long training—the competency of these oxygen measurements to indicate the effect of the phosphorus deficiency on the heat production seems questionable.

A recent paper by Robbins ('35) reports that with a 37% increase in the basal metabolic rate, under the influence of dinitrophenol, there was no significant change in the excretion of calcium, phosphorus and nitrogen. This was taken to

signify that increased metabolism of the body, as a whole, is not necessarily shared by the skeleton—at least in so far as changes in the intrinsic metabolism of the bones are measurable by alterations in the total exchange of the bone salts.

Aubel, Hughes and Lienhardt ('36 a, b) conducted experiments in a study of the effects of the plane of phosphorus intake on the gain in weight and body measurements of swine, the food consumption being maintained the same, in the high-phosphorus and the low-phosphorus groups, by permitting the pigs to have water only as it was consumed mixed with the feed.

From this study the authors concluded that the following abnormalities may result from the feeding of low-phosphorus rations: 1) a lowering of the inorganic phosphorus in the blood, 2) a failure of normal growth and development of bone and muscle, 3) a poor utilization of feed and storage of energy, 4) a loss of appetite, and 5) a marked increase in thirst, and a corresponding excretion of urine.

In speaking of the effects of phosphorus deficiency on the kidneys the authors said,

> Microscopically this organ showed evidence of a chronic diffuse nephritis of the parenchymatous type, and presented widened glomerular spaces around the glomerular tufts, and also widely distended uriniferous tubules with flattened epithelial cells.

The conclusion as to the utilization of food and storage of energy rested upon live weight data, and other observations, without body analyses or chemical study of metabolism.

Aubel and associates ('36 a) call attention to the fact that in the experiments of Riddell, Hughes and Fitch phosphorus deficiency in cows led to increased water intake, and increased outgo of urine.

Kleiber, Goss and Guilbert ('36) conducted a feeding and energy metabolism study of phosphorus deficiency as affecting beef heifers. There was an unfavorable effect of phosphorus deficiency on the appetite; a decrease in the inorganic phosphorus of the blood; but no effects on the body

temperature, the digestibility and the metabolizability of the food energy, and the fasting katabolism.

These authors concluded that phosphorus deficiency decreased the efficiency of food protein to spare the katabolism of body protein; and decreased the efficiency of utilization of food energy consumed in excess of the maintenance requirement.

From the data presented it is not possible to judge as to the validity of the important conclusion last stated; in fact, though food phosphorus must be necessary to the normal utilization of food energy and protein, in accord with the prevailing understanding of the fundamental physiology of nutrition, there remains a decided lack of clear-cut evidence that variations in the phosphorus content of the diet, under conditions of practical nutrition, affect the efficiency with which the food is utilized.

The investigation to be discussed comprised two experiments, which will be designated experiment 1, and experiment 2, the details of technic in both being as outlined by Swift, Kahlenberg, Voris and Forbes ('34). In both experiments comparisons were made of two rations differing only in phosphorus content, by means of 10 weeks' growth, metabolism and body analysis studies, in which the feed was apportioned by the paired litter-mate feeding method.

EXPERIMENT 1

The subjects were twelve pairs of weanling albino rats; six pairs being males, and six pairs females.

The basal ration consisted of wheat gluten 14%, casein (vitamin-free) 8%, Crisco 10%, dextrin 59.16%, salt mixture xxx 2.60% and cod liver oil 5%. The salt mixture was a modification of Osborne and Mendel's formula, free from calcium and phosphorus (Sherman and Smith, '31). In order to have a proportion of calcium equivalent to that found in synthetic rations including the usual Osborne and Mendel complete salt mixture, 1.24% of calcium carbonate, c.p., was added to one ration, and 1.28% tricalcium phosphate, c.p.,

to the other ration. In the phosphorus-supplemented ration the dextrin was reduced to 59.12%. Vitamins B and G, and additional cystine, were furnished to all rats by feeding 0.20 gm. of dried brewer's yeast daily.

The supplemented ration contained 0.366%, and the phosphorus deficient ration of 0.137%, of phosphorus.

Thus an average weekly food consumption of 40 gm. of the basal part of the low-phosphorus ration furnished 54.8 mg. of phosphorus; and, with 1.4 gm. of yeast (2.49% phosphorus), the deficient rats obtained a total of 89.7 mg. of phosphorus per week.

A diet fed for 8 or 9 days at the beginning of the experiment, but which proved unsatisfactory, and was therefore changed, differed from that above specified in that blood albumin was used in the place of the casein. The ration was analyzed, and was accounted for in the computation of the intake of phosphorus, protein and energy.

At the end of the experiment, the rats were killed, and x-ray photographs were taken of one hind leg of each individual. The bodies were then analyzed for moisture, ether extract, nitrogen, energy and phosphorus.

In view of the fact that the results corresponding to the two diets were essentially the same in regard to so large a proportion of the observations made, the numerical findings are presented only in summary form, as in tables 1 and 2.

After the termination of this experiment a special test of the palatability of the diet used was made by means of five pairs of rats, fed ad libitum, with measured feed intake and live weight, during 5 weeks. During 4 of the 5 weeks the rats ate more of the deficient than of the supplemented ration, and the total amount of the deficient ration eaten was 6.8% more than that of the supplemented diet, the natural inference being that the calcium phosphate served to diminish the palatability of the diet in which it was fed.

DISCUSSION OF RESULTS

In this experiment there were 358 refusals of feed, 206 being by the animals on the phosphorus supplemented diet. This deviation of 27.0 from the mean, if chance only determined the result, is 2.85 times the standard deviation, 9.46, and would occur by chance once only in 230 trials. The phosphorus-supplemented ration, therefore, limited the food consumption of the rats on the phosphorus deficient ration.

The average gains in body weight (contents of alimentary tract removed) of the rats on the phosphorus deficient and phosphorus supplemented rations were 90.15 gm. and 89.87 gm., respectively, this difference not being statistically significant. The growth for the 10 weeks was fairly satisfactory.

The fact that the supplemented diet was the limiting factor in relation to food consumption, and that there was practically no difference in the live weights of the rats fed the two rations, suggests that the phosphorus content of the basal diet, low though it was, was sufficient to satisfy the requirements of the fundamental physiological functions.

The data presented in table 1 show that the difference between the phosphorus contents of the diets (0.137 and 0.366%) produced a highly significant difference between the phosphorus contents of the bodies of the two groups (0.94 and 1.08%); but that this difference in food phosphorus produced no statistically significant difference between the phosphorus deficient and the phosphorus supplemented groups with reference to any other of the important observations indicating the relative economy of utilization of food energy and protein.

The x-ray photographs revealed no discernible difference in the bones of the two groups, though the skeleton was doubtless the seat of the greater part of the difference in the phosphorus contents of the bodies; and these photographs also revealed the fact that the phosphorus deficient rats were not rachitic.

EXPERIMENT 2

In this experiment efforts were made to obtain better food consumption and growth by changes relating to both rats and diets; thus ten pairs of male, pied rats were used instead of albinos; the rats were allowed to attain a weight of about

TABLE 1

Comparative utilization of food by rats on phosphorus deficient and phosphorus supplemented diets

	PHOSPHORUS DEFICIENT DIET	PHOSPHORUS SUPPLEMENTED DIET	ODDS THAT DIFFERENCE IS SIGNIFICANT
1. Ratio of gain in weight to dry matter of food	25.4	25.4	..
2. Energy of body gain as percentage of food energy	11.6	11.0	4 to 1
3. Food nitrogen retained, per cent	26.7	27.2	2 to 1
4. Energy gained as fat, per cent (of gain)	43.7	40.5	9 to 1
5. Energy gained as protein, per cent (of gain)	56.3	59.5	9 to 1
6. Digestibility of food protein, per cent	95.3	95.1	12 to 1
7. Digestibility of food energy, per cent	96.3	96.3	..
8. Heat production as per cent of food energy	81.1	81.9	15 to 1
9. Phosphorus content of rat bodies, grams	0.94	1.08	10,000 to 1

NOTE: All data in the above table are averages of individual values representing twelve rats.

120 gm. before the experiment was started, whereas in experiment 1 the albino rats were used as at weaning; and, in relation to the diets, the wheat gluten component was diminished from 14% to 6%; 8% of casein was replaced by 12% of lactalbumen; 10% of Crisco was replaced by 15% of butterfat; and a vitamin B concentrate was added to the diet.

The components of the diets were as follows:

	Phosphorus deficient	*Phosphorus supplemented*
Wheat gluten	6.0	6.0
Lactalbumen	12.0	12.0
Dextrin	60.7	60.7
Butterfat	15.0	15.0
Salt mixture	2.6	2.6
$CaCO_3$	1.4	1.4
$NaHCO_3$	2.7	..
Na_2HPO_4	..	2.3
	100.4	100.0

In addition, vitamin-containing amendments, as follows, were fed apart from the remainder of the diet, each day, to the rats of both groups: cod liver oil 12 drops (0.35 gm.); Harris yeast concentrate 1 tablet (0.1107 gm.), and diluted vitamin B concentrate [3] 12 drops (0.53 gm.; equal to 4 drops vitamin B complex liquid, type 1; equal to 0.1767 gm. dry matter).

The potency of this vitamin B complex was given by the manufacturer as B_1, 40 Chase and Sherman units per cubic centimeter, and B_2, 6 Sherman and Borquin units per cubic centimeter. This was fed in a 1 to 2 (H_2O) dilution.

The phosphorus deficient diet contained 0.133%, and the phosphorus supplemented diet 0.653% phosphorus—on an average—the percentages differing slightly for the different individuals, since the vitamin supplements were fed in constant quantities separate from the remainder of the diet.

The effective palatability of the diets, as indicated by the refusal of feed, as the feed for each pair was increased until one individual refused, was, in this experiment, in favor of the supplemented diet. In eight among the ten pairs of rats the individual which received the supplemented diet refused feed a smaller number of times than did its pair mate; and among a total of 357 refusals of feed there were 124 with the supplemented diet, and 233 with the deficient diet. Whatever the influence of the calcium phosphate as an unpalatable component of the diet in the first experiment, therefore, this was

[3] Vitab Products, Inc., San Francisco.

counterbalanced by the effects of the several changes that were made in the diet between the first and the second experiments.

During the course of the experiment it was observed that the feces from the deficient diet were much darker in color than those from the supplemented diet, and rat no. 5 passed bloody urine, after having been on the deficient diet 19 and 20 days. As the animals were killed, at the end of the experiment, the contents of the intestines of the deficient rats were, as a rule, much darker than were those of the phosphorus-supplemented rats. The small intestines of the deficient rats were black, as compared with the dark, yellowish gray intestines of the supplemented rats; and the large intestines of the former were also nearly black, as compared with the normal, dark greenish color of the intestines of the supplemented rats.

Also, with rats nos. 1, 3, 5 and 9 one kidney was enlarged, and soft, and contained cheesy products of tissue destruction; while in rats nos. 1, 3 and 5 the liver was small, and dark in color. The one abnormal kidney in a supplemented rat was in no. 16. In this case the medulla had completely degenerated—only the cortex remaining. The significance of these observations was not determined.

During the first 4 weeks the rats were fed once a day; but, beginning with the fourth week, they were fed twice daily, with the hope that this would lead to increased food consumption.

The basal metabolism was determined by the Haldane open-circuit method, once, on each rat, during the quiet part of 5- or 6-hour intervals between 8.00 or 9.00 o'clock in the morning and 2.00 or 3.00 o'clock in the afternoon, between the sixth to the eighth weeks of the experiment.

The numerical data, as presented in table 2, reveal significant differences between the groups of rats on the two diets only with reference to the digestibility of the food protein, which was the higher with the rats on the deficient diet, and with reference to the phosphorus content of the bodies of the rats.

While the effect of the phosphorus supplement to depress the digestibility of the protein of the diet was slight, this tendency was manifest in nine among ten pairs of rats, and the odds were 216 to 1 that the digestibility of the protein of the supplemented diet was less than that of the deficient diet. However, since the food consumption of each pair of rats

TABLE 2

Comparative utilization of food by rats on phosphorus deficient and phosphorus supplemented diets

	PHOSPHORUS DEFICIENT DIET	PHOSPHORUS SUPPLEMENTED DIET	ODDS THAT DIFFERENCE IS SIGNIFICANT
1. Ratio of gain in weight to dry matter of food	9.6	10.1	3:1
2. Energy of body gain as percentage of food energy	5.4	6.2	4:1
3. Food nitrogen retained, per cent	17.9	18.8	2:1
4. Energy gained as fat, per cent (of gain)	35.7	40.8	4:1
5. Energy gained as protein, per cent (of gain)	64.3	59.2	4:1
6. Digestibility of food protein, per cent	93.1	92.3	216:1
7. Digestibility of food energy, per cent	95.5	95.2	18:1
8. Heat production as per cent of food energy	85.6	86.2	4:1
9. Basal metabolism, calories per 100 gm. body weight	575.5	553.7	18:1
10. Phosphorus content of rat bodies, grams	0.98	1.16	2499:1

NOTE: All data in the above table are averages of individual values representing ten rats.

was determined by the individual that desired the smaller quantity, the food intake of the ten pairs was not exactly the same, and the two groups of digestion coefficients compared were not exactly homogeneous as to significance.

The range of variation in the digestibility of the protein of the two diets was as follows: for the deficient diet, 91.8 to 94.2%; and for the supplemented diet 90.6 to 93.2%.

The phosphorus content of the bodies of the rats differed consistently in accord with the phosphorus contents of the diets, the average difference being 18.37% of the quantity found in the bodies of the rats on the phosphorus deficient diet.

SUMMARY

The effects of phosphorus deficiency on the growing rat were studied in two 70-day body balance and metabolism experiments conducted with paired feed control.

In the first experiment the phosphorus deficient and the phosphorus supplemented diets contained 0.137% and 0.366% of phosphorus, respectively, on the dry matter basis; and after two groups of rats had consumed these diets for 70 days the corresponding average phosphorus contents of their bodies were 0.94% and 1.08%, respectively, the difference between these values being 15% of the lower one.

In the second experiment the low and the high phosphorus diets contained 0.133% and 0.653% of phosphorus, respectively; and after 70 days' feeding on these diets the corresponding average phosphorus contents of the rats' bodies were 0.98% and 1.16%, respectively, the difference between these values being 18% of the lower one.

In the first experiment, the difference in dietary phosphorus intake which produced a 15% difference in body phosphorus produced no observed difference in growth or in the utilization of food energy or protein.

In the second experiment, the difference in phosphorus intake which produced an 18% difference in body phosphorus produced a slight but statistically significant depression in the digestibility of food protein. This was the effect of the disodium phosphate of the high-phosphorus diet.

In this second experiment there were no other observed effects of the difference in phosphorus intake on the utilization of food energy or protein, or on growth.

The phosphorus content of the low phosphorus diet was as low as the experimenters could make it and at the same time have sufficient feed consumption and growth to serve the purposes of the investigation. Normal growth could not have been obtained on a diet still lower in phosphorus.

LITERATURE CITED

AUBEL, C. E., J. S. HUGHES AND H. F. LIENHARDT 1936 a The effects of low-phosphorus rations on growing pigs. J. Agric. Res., vol. 52, pp. 149–159.

———— 1936 b Phosphorus requirements in the ration of growing pigs. Kansas Agric. Exp. Sta., Tech. Bull., no. 41, 86 pp.

ECKLES, C. H., R. B. BECKER AND L. S. PALMER 1926 A mineral deficiency in the rations of cattle. Minn. Agric. Expt. Sta., Bull. no. 229, 49 pp.

ECKLES, C. H., AND T. W. GULLICKSON 1927 The relation of phosphorus deficiency to the utilization of feed. Am. Soc. Anim. Prod., Proc., pp. 16–25.

ECKLES, C. H., T. W. GULLICKSON AND L. S. PALMER 1932 Phosphorus deficiency in the rations of cattle. Minn. Agric. Exp. Sta., Tech. Bull., no. 91, 118 pp.

KLEIBER, M., H. GOSS AND H. R. GUILBERT 1936 Phosphorus deficiency metabolism and food utilization in beef heifers. J. Nutrition, vol. 12, pp. 121–153.

RIDDELL, W. H., J. S. HUGHES AND J. B. FITCH 1933 The energy metabolism of phosphorus deficient dairy cattle. Am. J. Physiol., vol. 106, p. 676.

———— 1934 a The influence of phosphorus deficiency in dairy cows on the coefficient of digestibility and the balance of calcium and phosphorus. J. Agric. Res., vol. 48, p. 167.

———— 1934 b The relation of phosphorus deficiency to the utilization of feed in dairy cattle. Kans. Agric. Exp. Sta., Tech. Bull. no. 36, 54 pp.

ROBBINS, C. L. 1935 The effect of dinitrophenol on calcium and phosphorus metabolism. J. Nutrition, vol. 10, pp. 185–191.

SHERMAN, H. C., AND S. L. SMITH 1931 The Vitamins, 2nd ed. The Chemical Catalog Co., Inc., New York.

SWIFT, R. W., O. J. KAHLENBERG, L. VORIS AND E. B. FORBES 1934 The utilization of energy-producing nutriment and protein as affected by individual nutrient deficiencies. I. The effects of cystine deficiency. J. Nutrition, vol. 8, p. 197.

THEILER, A., H. H. GREEN AND P. J. DU TOIT 1924 Phosphorus in the live-stock industry. Union So. Africa, Dept. Agric. J., vol. 8, p. 460.

WOODMAN, H. E., AND R. E. EVANS 1930 Nutritive value of pasture. VI. The utilization by sheep of mineral deficient herbage. J. Agric. Soc., vol. 20, p. 587.